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Editorial Views and News

The Thirty-ninth General Meeting of the International Association for Dental Research has come and gone. It proved to be a rather intensively earnest gathering. This was the first time the annual meeting was held in Boston. Registration totaled 894, as contrasted to 820 in Chicago last year and 687 in San Francisco in 1959. The yearly increase in attendance has been gratifying, but one must keep in mind that the membership has also increased every year. Moreover, at the Boston meeting 181 individuals were elected to membership at the final business session, Sunday, March 26, 1961.

One of the significant events at each IADR annual meeting is the Saturday-evening banquet. At this event the President-Elect, James A. English, now Dean of the School of Dentistry at the University of Buffalo, delivered an interesting dissertation entitled "International Aspects of Dental Research." As has become custom, this presidential inaugural address will be published in a future issue of the *Journal of Dental Research,* along with the proceedings of the meeting.

Awards recognizing meritorious work in research were presented as the banquet drew to a close. The annual Wilmer Souder Award was presented to Floyd A. Peyton, University of Michigan, for extended and commendable achievements in dental materials research. The Edward H. Hatton Award was given to the novice research worker judged to have presented the best paper at the current meeting. Seventeen persons competed in this eminent event this time. The award was presented to Jack G. Dale, Forsyth Dental Infirmary, for his paper on "The Use of Diffusion Chambers in Bone Resorption Studies." The runner-up was Eugene E. Fischer, Tufts University, for his paper on "Inhibition of Enamel Demineralization by Repeated Treatments with Sodium and Stannous Fluoride Solutions."

Conference on Dental Journalism.—This 1961 gathering was sponsored jointly by the Council of Journalism of the American Dental Association and the American Association of Dental Editors. This conference on March 13 and 14, held in the central office of the American Dental Association, took the form of a workshop built around the theme, "The Ideal Dental Journal." Participating editors from the United States and Canada were divided into six workshop groups. The Editor of this *Journal* had the privilege and responsibility of serving as chairman of the workshop on scientific articles. Each chairman reported back to the entire assembly the second day, when an intensive discussion period was held. This kind of conference certainly provided ample opportunity for profound deliberation as to the nature, value, and readability of various items, especially research reports which appear in the more significant dental journals of the land.

The New NIDR Building.—Since the dedicatory event is to occur very soon, it may be well to recall the history and objectives of this research institute.

Public Law 755, which formally established the National Institute of Dental Research in June, 1948, provided for the conduct and support of research on the causes underlying oral diseases and related conditions. This same legislation authorized funds for the construction of appropriate laboratory facilities to accomodate the newly created Dental Institute. However, the necessary funds of $2,000,000 did not materialize at this time. During the decade that followed, Congress, the USPHS, the American Dental Association, and the President of the United States, each indorsed the continuing need for appropriate dental-research facilities at the National Institutes of Health. During the same period, construction costs almost doubled, and the cost of the building which had been estimated in 1948 to be $2,000,000 had risen to $3,700,000 by 1958. Moreover, an appropriation of $100,000 provided in 1948 for architectural drawings had to be doubled eight years later.

Finally, on August 1, 1958, Dwight D. Eisenhower, as President, signed the appropriation bill for the Department of Health, Education, and Welfare, which included provisions for $3,700,000 to finance the construction of the long-awaited research building to house the Dental Institute. On the following page a photograph and descriptive information are provided for the record. The Editor and the *Journal* express the view that it is most gratifying to see such a significant edifice finally consummated.

—F. J. O.

FIG. 1.—The new Dental Research Laboratory, National Institutes of Health, Bethesda, Md.

DEDICATION OF THE NIDR

Indications are that the new home of the National Institute of Dental Research of the United States Public Health Service will be duly dedicated on May 20, 1961. Within the portals of this significant institution there will be conducted programs of basic and clinical research that the dental profession needs in order to understand more completely the nature of oral disease. It is hoped that from such research and comprehension of disease etiology there will emerge genuine and lasting solutions to the complex of oral disorders. The new laboratories will accommodate approximately two hundred researchers and will continue, as it has in the past, as a monument to the profession of dentistry. The total gross area in this building as pictured on this page will be 92,601 square feet; total volume, 999,400 cubic feet; and total net area, 41,582 square feet. The estimated cost is $3,777,798; estimated cost for fixed equipment, $715,000; and estimated cost for movable equipment, $760,000. The type of construction is reinforced concrete with cavity walls and brick-veneer exterior, beam-and-girder concrete-floor construction, and exposed concrete ceiling. The building comprises five stories above ground and two floors underground.

International Association for Dental Research
Abstracts of Papers Presented at the Seventh Annual Meeting of the Japanese Division

TOKYO DENTAL COLLEGE, TOKYO, OCTOBER 26, 1959

Compiled by MASAO ONISI

I. MASTICATION

1. PHYSIOLOGIC STUDIES ON THE MECHANO-RECEPTORS OF PERIODONTAL MEMBRANE.—*Mamoru Yamada, Sanya Sakada, Yoshio Murata, and Matsukichi Ueyama, Department of Physiology, Tottori University.* The responses of functionally single nerve fibers of sensory nerve endings of periodontal membrane evoked by mechanical stimuli (pressure and touch) were investigated in the canines of cats with the following results: (1) Slow- and fast-adapting units were recognized, and the former units were divided into two classes. (2) The range of response times of slow-adapting units by the maximum and minimum stimuli was *ca.* 100 msec.; the frequency of discharges was proportional to the logarithm of the pressure applied. (3) Touch receptors did not respond to pressure, but the pressure receptor of the fast-adapting unit responded to touch stimulus. (4) When repetitive stimuli were administered to the periodontal membrane, the peak of discharges occurred at a frequency of 100 c.p.s. Discharges were blocked at 250 c.p.s. The results suggest that the smallest interval is *ca.* 4 msec. in the mechano-receptor of the periodontal membrane.

2. BIOCHEMICAL STUDIES OF THE PERIODONTAL MEMBRANE ON THE STATIC STUDIES OF PROTEIN COMPONENT.—*Shimpei Araya, Toshiro Ishiwara, and Shigeru Saito, Departments of Prosthetic Dentistry and Biochemistry, School of Dentistry, Tokyo Medical and Dental University.* Electron-microscopic observations have demonstrated that the periodontal membrane consists principally of typical collagen fibers having 640 Å periods. In the periodontal space, the collagen fibers are formed fairly rapidly and may be destroyed with similar rapidity. Samples of fresh bovine periodontal membrane were extracted five times with 0.2 M Na_2HPO_4 solution at hourly intervals (extracted protein: 4.2 per cent), followed by extraction with McIlvaine buffer (pH 4) five times at 24-hour intervals at about 0°–4° C. Fibers were reprecipitated from the final extract by changing the pH and ion strength. These fibers showed 640 Å periods, the same as those observed in the natural periodontal fiber. The components of all extracts and residues were determined. Free hydrolyzed amino acids, particularly proline, hydroxyproline, arginine, and tyrosine, were quantitatively measured, and sugar, including amino sugar, was estimated.

3. HISTOLOGIC STUDY ON SHARPEY'S FIBERS IN THE CEMENTUM.—*Masatoyo Akizoshi and Wasayuki Inoue, Department of Oral Pathology, School of Dentistry, Tokyo Medical and Dental University.* Histological investigations were carried out on Sharpey's

fibers in the primary and secondary cementum of tangential sections taken from the cervical and apical regions of 13 functioning, 15 non-functioning erupting, and 4 impacted teeth. The following results were obtained: (1) In the functioning teeth of young adults, Sharpey's fibers in the primary cementum were polygonal on transverse section and measured 4–6 μ in diameter. Twenty or 30 per 1,000 μ^2 were counted. No significant differences in size and number of fibers in various parts of the cervical and apical regions were observed. The secondary cementum contained round fibers that were usually smaller and fewer in number. (2) In the non-functioning erupting teeth of infants, the polygonal fibers in the primary cementum were as small as 2–4 μ, and as many as 50–100 fibers per 1,000 μ^2 were observed. (3) In the impacted teeth, Sharpey's fibers in the primary cementum appeared more slender and fewer in number, as in the non-functioning erupting teeth, and much smaller numbers of them were observed in the secondary cementum.

4. An Artificial Compound for Use in the Evaluation of Masticatory Efficiency.—*Michiyasu Sato and Seizo Yokota, Department of Orthodontics, Kyushu Dental College.* Since the search for the proper material to be used conveniently for testing human masticatory functions has heretofore proved unfruitful, an attempt was made to synthesize a compound that would fulfil the need as nearly as possible. Of numerous compounds so far tested, the one prepared in tablet form with barium sulfate as base and with an equal quantity of carbayba wax as molding material proved most fitted to the requirements. Preliminary experimentation on this compound conducted under compression and impact crushing methods yielded the following results. The pulverizability of this compound conformed closely to the empirical formula of Rosin and Rammler as represented by the exponential equation,

$$y(x) = c^{-bx^n},$$

where y = cumulative percentages oversize, x = diameter of particle, c = base of natural logarithm, and b and n are coefficients of the material concerned. Second, a question was raised as to how the above equation could be applied in expressing the masticatory efficiency using the present compound, in which, with an increasing number of crushing strokes, the value n decreased while the value b increased. Since these concurrently shifting values in the equation obviously made a comparison of masticatory efficiency difficult, a new device was introduced to combine the two values into a single value by which the masticatory efficiency could be successfully expressed, and this figure was named "M.C." The results of applying the new method for evaluating masticatory efficiency have confirmed its great utility and low error of measurement.

5. Masticatory Efficiency in Subjects with Normal Occlusion.—*Taro Ueda and Seizo Yokota, Department of Orthodontics, Kyushu Dental College.* The synthetic test compound designed by Sato (see preceding abstract) was applied to the human masticatory apparatus, using 5 males and 5 females from nineteen to thirty-one years of age exhibiting normal tooth occlusion. Masticatory efficiency was evaluated by measuring the particle size distribution after the compound was subjected to a certain number of masticatory crushings. It was revealed that the relation between the value y, representing cumulative percentage oversize, and the value x, representing particle diameter, could be expressed by the equation

$$y(x) = c^{-b_1 x^n}, \qquad (1)$$

where b_1 and n are coefficients. The relation between the value y and the value z, representing number of masticatory strokes, could be represented by the equation

$$y(x) = c^{-b_2 z^m}, \qquad (2)$$

where b_2 and m are coefficients. In equation (1), when the log(-log) scale of cumulative percentage oversize was plotted on the x-axis, the curve of particle size distribution was linear. In equation (2), when the log(-log) scale of cumulative percentage oversize was given on the y-axis and the log scale of number of masticatory strokes was given on the x-axis, the curve of masticatory crushing became linear. In these graphs, the line on 36.8 per cent, drawn parallel to the x-axis, was chosen as the standard criterion for evaluating masticatory efficiency.

6. Brain-Stem Mechanism Controlling the Jaw Muscle Tonus in the Dog.—*Yojiro Kawamura, Masaya Funakoshi, and Mitsuru Takata, Department of Oral Physiology, Dental School, Osaka University.* This is a study of a "feedback" mechanism in the brain stem controlling the function of the masticatory muscles. Points in the brain stem responding to the expansion of each masticatory muscle were determined electrophysiologically, and the character of the responses of each point was analyzed. Each masticatory muscle was associated with an individual point of the trigeminal motor nucleus. Reciprocal functional relations were observed between the two trigeminal motor nuclei and the hypoglossal nucleus.

7. Bruxism.—*Yasuhide Takahama, Department of Orthodontics, Tokyo Medical and Dental University.* The mechanisms of bruxism were investigated by means of an electromyogram, electroencephalogram, electrocardiogram, respiratory curve, and sound wave during sleep in seven subjects. EMG revealed that the masticatory muscles on both sides are rhythmically alternated when bruxism occurs. Individual masticatory muscles could be identified by their particular patterns of rhythmic activity whenever bruxism occurred. These patterns were not changed by the administration of chlorpromazine, meprobamate, or morphine chloride. No rhythmic activity of those muscles was observed when subjects ground their teeth while awake. EEG revealed that bruxism usually occurs during light sleep. No special tendency among these subjects could be seen in the patterns of sleep curves. Rhythms of pulse and respiration were changed, and voluntary discharges were found in masticatory and wrist muscles before bruxism. Brain waves showed changes which were considered as evidence of increased activity of the cortex before these changes occurred. During bruxism the pulse rate increased, and respiratory movement during inspiration was inhibited and became irregular. No special order in the occurrence of changes in EEG, EMG, and respiratory curve was observed. The above-mentioned changes suggest that bruxism is closely related to the autonomic nervous system. No definite relationship between bruxism and the masticatory process was observed.

8. Neural Mechanisms of Gnashing.—*Yojiro Kawamura, Shusaku Tsukamoto, and Kiyokatsu Miyoshi, Department of Oral Physiology, Dental School, Osaka University.* The neural mechanism of gnashing was studied in rabbits. The authors succeeded in inducing gnashing in rabbits by electrical stimulation of the brain cortex. High-frequency electrical stimulation of the jaw motor area of the cortex induced the characteristic gnashing and did not induce any other body movement. The characteristics of gnashing were analyzed.

9. Dynamic Study of Attachments.—*Shohei Homma, Masami Homma, and Yasuo Nakarmura, Department of Dentistry, School of Medicine, Keio University, and Mamoru Yamada, Department of Physiology, School of Medicine, Tottori University.* A dynamic study was made of the distribution of force exerted on the abutment tooth through the measurement of boundary and principal stress. The investigation employed a photoelastic method, using attachment and inlay abutment. Materials were Cheyes' and modified Okada's precision attachments, modified Black class II inlay abutment, which had a rention groove and whose gingival wall was oblique (Okada's about 15°, Tylman's about 45°), and lower first molar as an abutment tooth. (1) The inlay attachment did not show any unfavorable effect in comparison with a class II inlay for restoration. (2) Modified Okada's attachment is stable, because its principal stress line is along the long axis of the tooth. (3) The combination of Okada's inlay abutment and modified Okada's attachment showed the best functional effect as an attachment system. Dynamic considerations should be emphasized in tooth restoration, in view of the pathologic effect of stress in relation to periodontal disease, axial rotation of the tooth, and mandibular deformation.

II. ORAL MUCOSA

10. Permeability of the Oral Mucous Membrane. III. Permeability to Amino Acids and Propylene Glycol and Biophysiologic Characteristics of the Oral Mucous Membrane.—*Yojiro Kawamura and Mitsuru Takata, Department of Oral Physiology, Dental School, Osaka University.* It has been reported that various chemical substances will penetrate through the oral mucous membrane by simple diffusion. However, the basic mechanism of this phenomenon has not yet been determined. The permeability of potassium and sodium ions and the impermeability of calcium ions across the oral mucous membrane were previously detected by the authors, and the oxidative process of the membrane has also been proved to have an intimate relation to such an ionic transport. In this experiment, whether amino acids can permeate the oral mucous membrane was determined, and the biophysiologic characteristics of the oral mucous membrane were considered. Sublingual mucous membrane of a dog was used throughout the experiment. The experimental procedures were similar to those reported in our previous paper. Glycine and histidine dissolved in physiologic saline solution were used. The manometric method was used for the quantitative analysis of the amino acids. Neither the glycine nor the histidine passed across the oral mucous membrane, but these amino acids were decreased in the outer medium, indicating partial incorporation into the membrane. After the mucous membrane was treated with cationic or anionic detergents, however, the amino acids were able to pass through them, but no transport of amino acids was observed after treatment with a non-ionic detergent. On the other hand, acetylcholine penetrated through the sublingual mucous membrane when it was dissolved in propylene glycol, and it affected the electrical activity of the tongue muscle. These results suggest that the lipoprotein in the mucous membrane prevents the transference of chemical substances across the oral mucous membrane.

11. Exfoliative Cytology of Mouth Washings and Garglings.—*Yoshio Watanabe and Yohio Shiraishi, Department of Oral Surgery, Okayama University*

Medical School. Exfoliative cytologic study of the oral cavity has been carried out by using smears taken directly from the surface of the mucosa, but not from washings or garglings. Preliminary studies were made to determine the effective centrifuging factors in collecting exfoliated cells. Subsequently, 83 normal cases and 76 pathologic cases were investigated, including 25 cases of newborns and sucklings, 30 adults, and 28 edentulous old persons with normal healthy oral cavities, 24 malignancies, 6 benign tumors, 5 various other oral lesions, and 41 smokers. Materials were collected by means of centrifugation, the membrane filter technique, and the cell-block method. All the specimens were stained by the Papanicolaou technique. It was concluded that examination by centrifugation of mouth washings or garglings is simple and the cells easy to collect, causes little irritation to the oral tissue, and can be repeated many times. It is, therefore, a very satisfactory method to use for observing postoperative progress in which a remnant of malignant tissue is suspected, for examining cells after radiation therapy, and for the early detection and examination of extensive lesions of the oral mucosa.

12. The Relationship of Squamous-Cell Volume, Lactobacillus, Streptococcus, and Veillonella Counts in the Human Mouth.—*Wataru Kondo and Masao Onisi, Department of Oral Microbiology, Tokyo Medical and Dental University.* Sediment in peptone water with which the mouth had been flushed was separated into three fractions by means of hematocrit tubes and centrifuge. The first fraction, centrifuged at 840 r.p.m. for 3 minutes, consisted mainly of desquamated epithelial cells, and the second fraction, separated from the remaining supernatant by centrifugation at 2,000 r.p.m. for 2 minutes, consisted of naked nuclei and salivary corpuscles. These two fractions made up most of the sediment. The third fraction, consisting of microorganisms, was thus negligible with respect to the volume. The cell volume and the respective numbers of lactobacilli, streptococci, and veillonellae from subjects with varying numbers of carious lesions were measured to determine whether there might be any relationships. The number of organisms was proportional to the epithelial cell volume, but not to the number of carious cavities. In addition, the count of veillonellae correlated more closely with that of streptococci than with lactobacillus counts.

III. HARD TISSUE

13. Sulfur-containing Lipids in the Hard Tissues.—*Shimpei Araya, Nobuhito Katsura, and Masaharu Shimizu, Department of Biochemistry, School of Dentistry, Tokyo Medical and Dental University.* The nucleic acid content in tooth buds was estimated according to the method of Rosen and Ogur. Lieberman-Burchard material, as well as sulfur, was found in the 70 per cent ethanol fraction. This finding led us to seek steroid sulfates in the hard tissues. Pulverized bovine bone was extracted with acetone by Sochlet's apparatus; the extract was dried and dissolved in water and was further extracted with *n*-butanol after washing with ether. This extract was then chromatographed. The chromatogram showed that the spots containing sulfur corresponded to those giving positive steroid tests not only with antimony trichloride, phosphomolybdic acid, and iodine but also with Zimmerman reaction and particularly with Ray's methylene-blue reaction for steroid sulfate. Further attempts are being made to isolate and purify these steroid sulfates.

14. In Vitro Pigmentation of the Dentinal Surface by Micro-organisms.—*Masao Onisi, Asako Yuge, and Kenshiro Takamori, Department of Oral Microbiology, Tokyo Medical and Dental University.* Pigmentation was observed to occur on the surface of tooth fragments which were incubated in test tubes for about 20 days at 37° C. under various conditions. Of a total of 86 strains of organisms representing 16 different genera tested in this study, more than half originated in the mouth. Preliminary study revealed that cultures of *Nocardia dentocariosa* and certain other oral bacteria grown in glucose broth readily pigmented tooth fragments and that the substance responsible for this known pigmentation in this medium was of extracellular origin, heat-stable, and filtrable through celloidin. More pronounced pigmentation appeared at lower pH's regardless of the strain used; it increased in amount at a pH as low as 4.0. Organisms which were incapable of lowering the pH did not cause pigmentation. However, adjustment of pH to produce acidity in such cultures resulted in pigmentation. Maintenance of an alkaline pH prevented pigmentation. Further studies have shown that meat extract, pyruvic acid, and soybean sauce can cause pigmentation under acidic conditions, while gelatin, peptone, acetone, and glucose cannot. Active substances, however, could be produced by repeated autoclaving of a peptone and glucose solution. Caramel was found to be the sole agent capable of staining in either alkaline or acid solutions. Partial decalcification of the tooth by EDTA seemed to be a necessary condition for staining. Every strain of organism tested could produce brown pigment when provided with a suitable medium from which meat extract was omitted.

IV. TUMORS

15. Histochemical Study of Adenoameloblastoma.—*Goro Ishikawa and Katsuyoshi Mori, Department of Oral Pathology, Tokyo Medical and Dental University.* Three cases of adenoameloblastoma were studied histochemically. Glycogen was demonstrated in various amounts in the epithelial cells and was most abundant in the cylindrical cells of the glandular structure. Masses in the ductlike structure were stained positively with PAS stain after salivary digestion. This substance also showed a positive reaction with mucicarmine stain and was metachromatic by toluidine blue at pH 4.1. However, the staining characteristics of these masses by van Gieson's, Mallory's, and Pap's silver stain resembled that of connective tissue. Continuity from the myxoid stroma to the ductlike structure was suggested in some areas. The origin of the ductlike structure, therefore, was not determined, although other findings suggested that it may develop in the parenchymal tissue because of unusual differentiation of the epithelial cells. Alkaline phosphatase reaction was weak in the epithelial cells of ameloblastomas, but the flattened epithelial cells adjoining cylindrical epithelium showed a positive reaction in the adenoameloblastoma. It is interesting to note that the cells of the stratum intermedium of the enamel organ were rich in phosphatase.

V. DENTAL MATERIALS

16. Influence of Clinical Variables on the Properties of a Cristobalite Investment.—*Takao Fusayama, Hiroyasu Hosoda, and V. M. Kher, Tokyo Medical and Dental University.* The influence of various clinical variables on the expansion and other properties of Shofu cristobalite investment was investigated. A water/powder

ratio of 0.35 was found most adequate. The ratio deviated from 0.35 to 0.37 when a plastic scoop was used for measuring powder. The range of variation in the total of setting and thermal expansions due to this deviation was smaller than 0.17 per cent. The addition of NaF to the mixing water effectively decreased the setting time and setting expansion and increased the thermal expansion little, whereas the addition of NaCl, $NaSO_4$, or KCl greatly increased the thermal expansion but roughened the mold walls. When the investment was heated within 3 hours of setting, the setting expansion, expected to continue, was incorporated in the thermal expansion measurement. Setting and thermal expansion were constant. Thus thermal expansion was slightly increased compared with the findings when heating was deferred for more than a day. Variation of heating rate from 10° C/30 sec to 10° C/3 min did not change the thermal expansion of the investment, but 10° C/15 sec seemed too rapid because of cracks. Cooling of a heated investment yielded a comparatively straight shrinkage curve, but reheating of it to the original temperature exactly reproduced the original thermal expansion.

Use of Roentgenograms and Lightgrams as Supplements to Autoradiographs of Tooth Sections

ROBERT E. GOING, MAURY MASSLER, and HAROLD L. DUTE

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Survey autoradiographs made on standard dental X-ray film for detecting the path of radioisotope penetration into tooth structures or around filling margins have not been completely satisfactory because of the coarse grain and the difficulty in exactly locating the position of the isotope in relation to the tooth structure. The best method of achieving the exact location of the isotope in relation to the tooth structure is by direct tissue autoradiography, in which a fine-grain emulsion is placed directly on the surface of the tissue section.[1, 2] However, this requires a very thin tooth section, since in a thick section the radiations from isotopes deep in the section cause a blurred image. It is not possible to retain the filling *in situ* in very thin ground sections, so that dental X-ray film and photographic film remain the methods of choice in this area of dental research. Two techniques have been developed to supplement such autoradiographs. One technique utilizes X-ray radiations and the other light emissions to produce images of the tooth slab on X-ray film and thus aid in a more accurate location of the isotope.

The first superimposes a faint X-ray image of the tooth section over the autoradiograph, as suggested by Becks.[3] A comparison of Figure 1, *A*, with *B* shows that a sharper outline of the pulp, dentino-enamel junction, filling, and the entire tooth slab is obtained by the addition of the X-ray image.

After immersion of the tooth specimen in the isotope a plano-parallel center slab approximately 1 mm. (or less) in thickness was cut on a special machine. The tooth section was then placed (in the dark room) on a dental X-ray film (Minimax, extra fast) and pressed into tight contact with a plastic holder. The specimen was then placed in a light-tight container for a period of 24 hours, to produce a routine autoradiograph. However, before the film was removed for developing, the specimen in the light-tight box was taken to a dental X-ray machine and exposed to X-ray radiations for 4 seconds at 60 kvp, 10 Ma, at a 48-inch target distance. The film was then developed in the usual fashion. This caused an X-ray image of the tooth slab to be superimposed over the autoradiograph, as shown in Figure 1, *B*, and more accurately outlined the enamel, dentin, cementum, and pulp, as well as the margins of the filling, than was possible by the autoradiograph alone. The X-ray also tends to reduce slightly the halo caused by the long exposure to the isotope radiations.

The second technique produces a detailed and histologically accurate picture of the tooth slab on dental X-ray film (Fig. 2). This was done by exposing the specimen on the X-ray film to light instead of X-rays or isotope radiations.

The tooth slab was placed on a dental X-ray film (Minimax, extra fast) in the dark room as for an autoradiograph, but compressed lightly between two clean glass slides. A point source

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of light was used, mounted overhead at least 24 inches directly above the specimen. The specimen on the film was exposed in an otherwise completely dark room for approximately 0.4 second. The exposed X-ray film was then developed for 5 minutes, washed, and fixed. The resulting "lightgram" is shown in Figure 2, *B*.

Excellent details of the structural characteristics of the tooth slab are produced by this technique. It eliminates the need for more tedious and expensive photomicrographs and permits routine comparison of the autoradiograph with the specimen image. The sharpness of this image is greatly improved in thinner sections (compare Figs. 2, *B*, and 1, *C*).

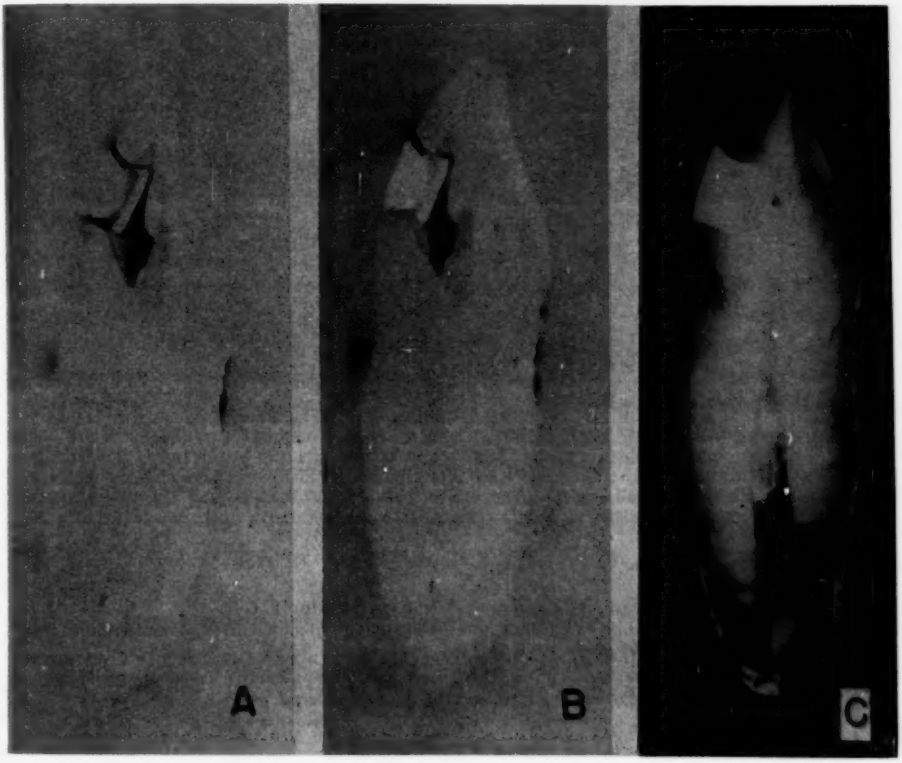

FIG. 1.—Class V silver amalgam restoration placed over a thick base of zinc phosphate cement in a freshly extracted lower incisor and immersed in a radioactive solution of $Na_2S^{35}O_4$ (10 microcuries/ml) for 24 hours. A central slab was then cut approximately 1.0 mm. in thickness. *A*, routine survey autoradiography (6 hours' exposure on extra-fast dental X-ray film); *B*, X-ray image of the tooth slab superimposed on a similar autoradiograph, *C*, a lightgram of the tooth slab. (Mag. 5.5.)

CONCLUSIONS

Analysis of autoradiographs made from ground sections of teeth can be improved by the addition of an X-ray image superimposed on the autoradiograph in the same film and by a lightgram which accurately depicts the structural characteristics of the ground section in a manner similar to a photomicrograph.

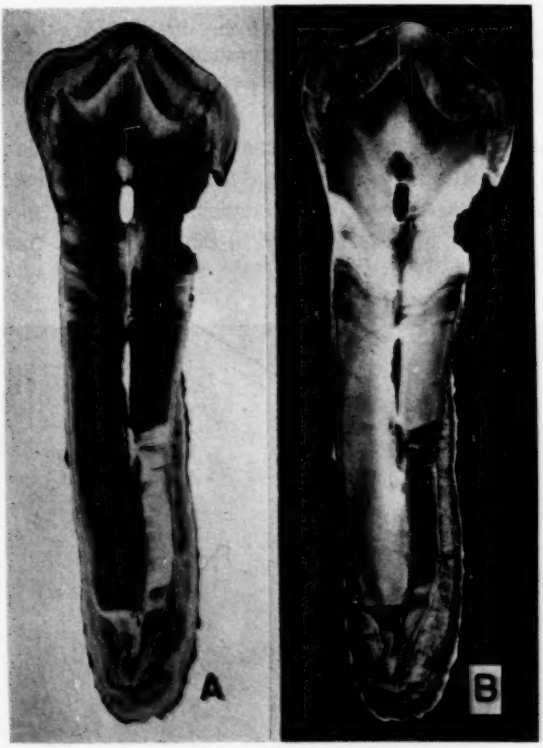

FIG. 2.—*A*, photomicrograph of a ground section approximately 120 μ in thickness; *B*, a lightgram of the same specimen. Note the almost perfect reproduction of histologic details. Note the carious lesions in the mesial and distal cervical areas, which were deeply pigmented in the original section, and the sclerotic dentin covered by secondary cementum in the apical area of the tooth. (Mag. 5.5)

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Electron-microscopic Observations on Soft Carious Human Dentin

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In the process of dentinal caries both the organic and the inorganic components of the tissue are ultimately destroyed and eliminated, with resulting cavitation. Since ultrastructural studies of dentin in various stages of decay should almost certainly contribute importantly to a better understanding of the carious process, an electron-microscopic survey of carious dentin was undertaken in our laboratories.

A number of papers have already appeared dealing with electron-microscopic observations on both artificially demineralized and undecalcified carious dentin. The distribution of micro-organisms in the carious lesion[1–5] and the presence of typical cross-striated collagenous fibrils in the matrix[1, 2] have been described. Lenz[6] found large calcified bodies in dentinal canals, but, to our knowledge, no detailed information concerning the mineral phase of carious dentinal matrix has been reported.

This paper deals with soft carious dentin presumed to be in an advanced state of decay. The character and distribution of the mineral moiety (apatite crystallites) of carious dentinal matrix are described. Dense mineral deposits lacking a collagenous matrix are also reported at the periphery of the dentinal canals. Finally, the appearance of the collagenous fibrillar component of carious dentinal matrix is described as it appears in sections stained with phosphotungstic acid and in homogenized tissue. The implications of certain of these findings as they relate to the carious mechanism are discussed.

MATERIALS AND METHODS

Permanent human teeth with apparently active carious lesions were obtained from the Dental Clinic of Strong Memorial Hospital, Rochester, New York. The whole teeth or soft carious materials excavated from carious lesions were fixed immediately after operative removal in cold (4° C.) 1 per cent osmic acid solution containing sucrose (0.22 M) and buffered to pH 7.2–7.4 with 0.025 M veronal acetate buffer, in neutral 10 per cent formalin, or in 70 per cent alcohol. Some of the material was dehydrated in alcohol and imbedded in butyl methacrylate catalyzed with 1 per cent benzoyl peroxide. Some material was stored in 70 per cent alcohol and sectioned without further treatment. A few observations were also made on fresh, untreated tissue. Sections were cut with a microtome* equipped with a glass knife.

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* Porter-Blum.

For study of the organic phase, with particular emphasis on the collagenous matrix, sections were treated with 10 per cent phosphotungstic acid solution for periods ranging from 10 minutes to 2 hours; this treatment had the effect of simultaneously decalcifying the tissue and staining the collagen. Untreated sections were used for study of the inorganic phase: in addition to the taking of routine electron micrographs, this study consisted of (1) dark-field observations to establish the crystalline nature of the particles identified as crystallites, (2) establishment of the apatite or apatite-like nature of these crystallites by electron diffraction, and (3) stereoscopic investigation of the three-dimensional morphology of the crystallites. The details of these latter observations are being published elsewhere as part of a larger treatment of crystallite morphology in dentin and bone.[7]

Because of the fact that stained sections contained no mineral matter and collagenous fibrils were not visible in unstained sections, a need was felt for preparations in which the collagenous and mineral components could be visualized simultaneously. An attempt to satisfy this requirement was made by preparing and observing specimens of formalin-fixed carious dentin homogenized* in distilled water. In this process, the tissue was exposed to distilled water for a period of 10–30 minutes.

RESULTS

General topographical description.—The typical morphologic pattern of dentin with dentinal canals and intercanalicular matrix was recognizable throughout the substance of soft carious dentin (Fig. 1) except in the vicinity of the external surface of the lesion, where dentinal substance was reduced to thin septa or trabeculae projecting into a mass of bacteria (Figs. 2, 3). The dentinal canals were considerably distended, and the volume of intercanalicular matrix was correspondingly reduced (Fig. 1). A discontinuous layer of variable thickness and consisting of densely calcified material formed a sort of incomplete lining of the dentinal canals. Large numbers of bacteria were present on the surface of the carious dentin and in the dentinal canals, where they completely filled the lumen (Figs. 1, 2, 4). Bacteria had also invaded the matrix, in which they appeared to be scattered singly in small lacunae or in groups occupying larger spaces (Figs. 1, 5–7). While no attempt was made to identify the various types of micro-organisms or to assess the frequency of individual species, it was noted that their distribution generally followed a tendency for a single type to predominate within individual tubules.

Mineral phase.—Unstained sections of undecalcified carious dentin were excellent material for studying the mineral moiety of the tissue; the non-mineral phase appeared homogenous, however, allowing none of its structural details to be discerned (Figs. 1, 2, 5, 6, 8–10). The mineral phase was in the form of separate thin platelike crystallites that presented thin, dense profiles when viewed on edge and broader, less dense profiles when viewed on their broad surface (Figs. 6, 8–10). These crystallites exhibited no uniformity of size or shape other than their general platelike structure; doubtless many of them represented vestiges (resulting from partial dissolution) or fragments (resulting from mechanical fracturing due to microtomy) of much larger cystallites. The thinnest profiles indicated a crystallite thickness of 35–40 Å, with maximum length of 800 Å. No pattern was discernible in the distribution or orientation of the crystallites. They appeared to be scattered more or less randomly throughout the matrix (Figs. 6, 8, 10).

* Dounce ground-glass homogenizer.

FIG. 1.—Low-power micrograph of a section of undecalcified soft carious dentin near superficial edge of lesion, showing oblique longisectional views of four dentinal canals (*C*). The rounded space (*D*), resembling a transverse section of a canal in the lower left, is probably a diverticulum of some canal. The canals are distended and filled with micro-organisms (*B*). The intercanalicular matrix is largely, but incompletely, decalcified by the carious process and contains some scattered miro-organisms (*B*). A heavily calcified discontinuous lining of the canals appears in most places as a black border at the edge of the intercanalicular matrix. The polystyrene latex spherules (courtesy of Dow Chemical Co.) are approximately 0.26 μ in diameter. (Mag. approx. 8,000.)

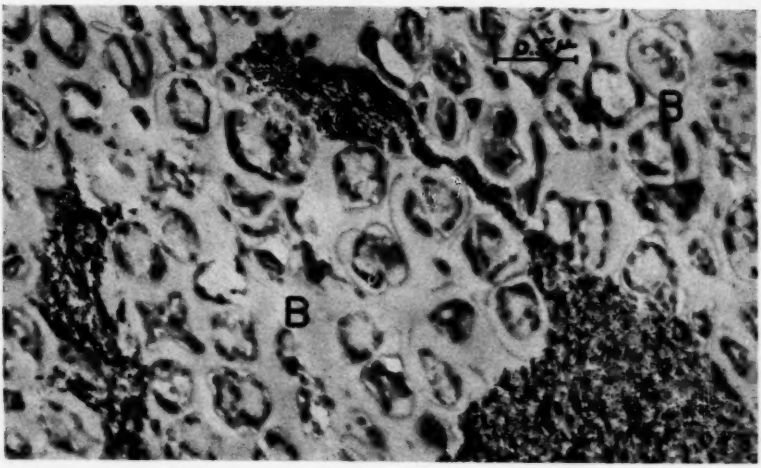

FIG. 2.—Undecalcified section of soft carious dentin. Two trabeculae of matrix are projecting into the mass of bacteria (*B*) at superficial edge of lesion. Individual crystallites appear as fine black specks. (Mag. approx. 23,000.)

FIG. 3.—Section of soft carious dentin decalcified and stained with phosphotungstic acid. A thin trabecula of intercanalicular matrix (*M*) is seen projecting into a mass of bacteria (*B*) at the superficial edge of the lesion. The collagen banding appears to be intact despite the proximity of the bacteria. (Mag. approx. 72,000.)

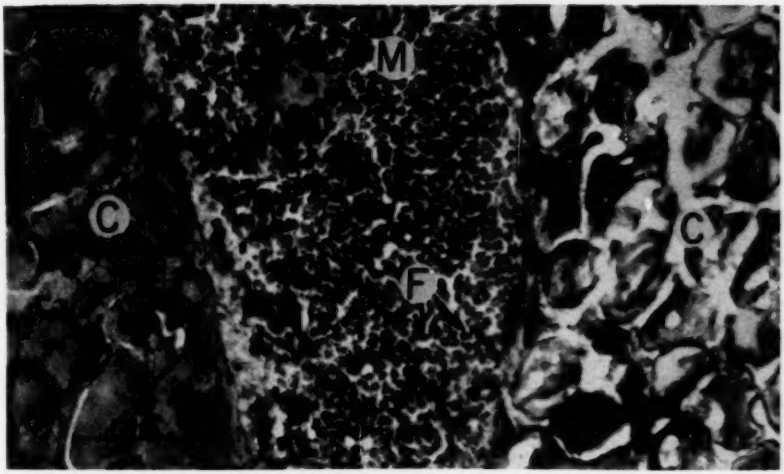

FIG. 4.—Section of soft carious dentin decalcified and stained with phosphotungstic acid. The picture shows micro-organism-containing dentinal canals (*C*) on each side, with intercanalicular matrix between them (*M*). The matrix is largely filled with collagenous fibrils seen in transverse section (*F*). (Mag. approx. 16,000.)

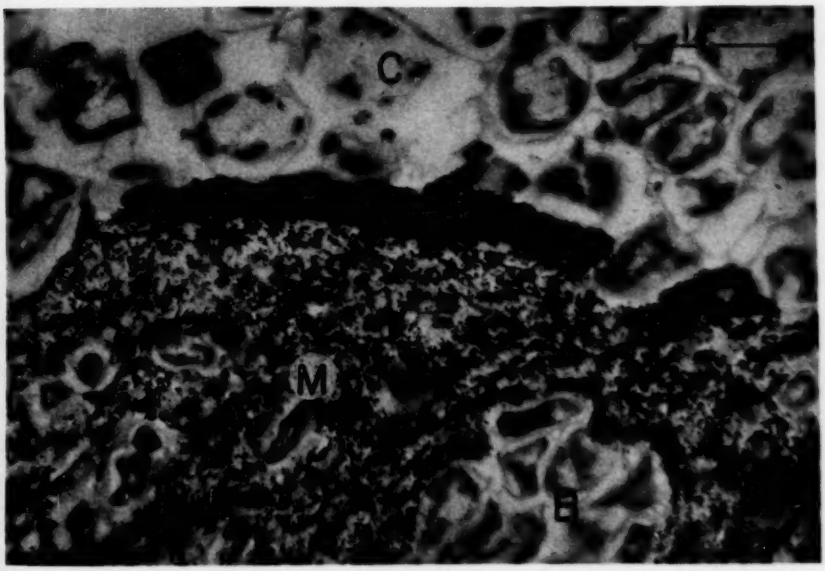

FIG. 5.—Undecalcified section of soft carious dentin. In the upper part of the picture is the lumen of a dentinal canal (*C*) filled with micro-organisms; in the lower part is the intercanalicular matrix (*M*), which contains some micro-organisms (*B*). Interposed between lumen and matrix is a densely calcified (*black*) layer. Note that breaks in the lining contain bacteria. (Mag. approx. 20,000.)

FIG. 6.—Section of intercanalicular matrix (*M*) of undecalcified soft carious dentin. Numerous micro-organisms (*B*) are seen in separate lacunae. (Mag. approx. 80,000.)

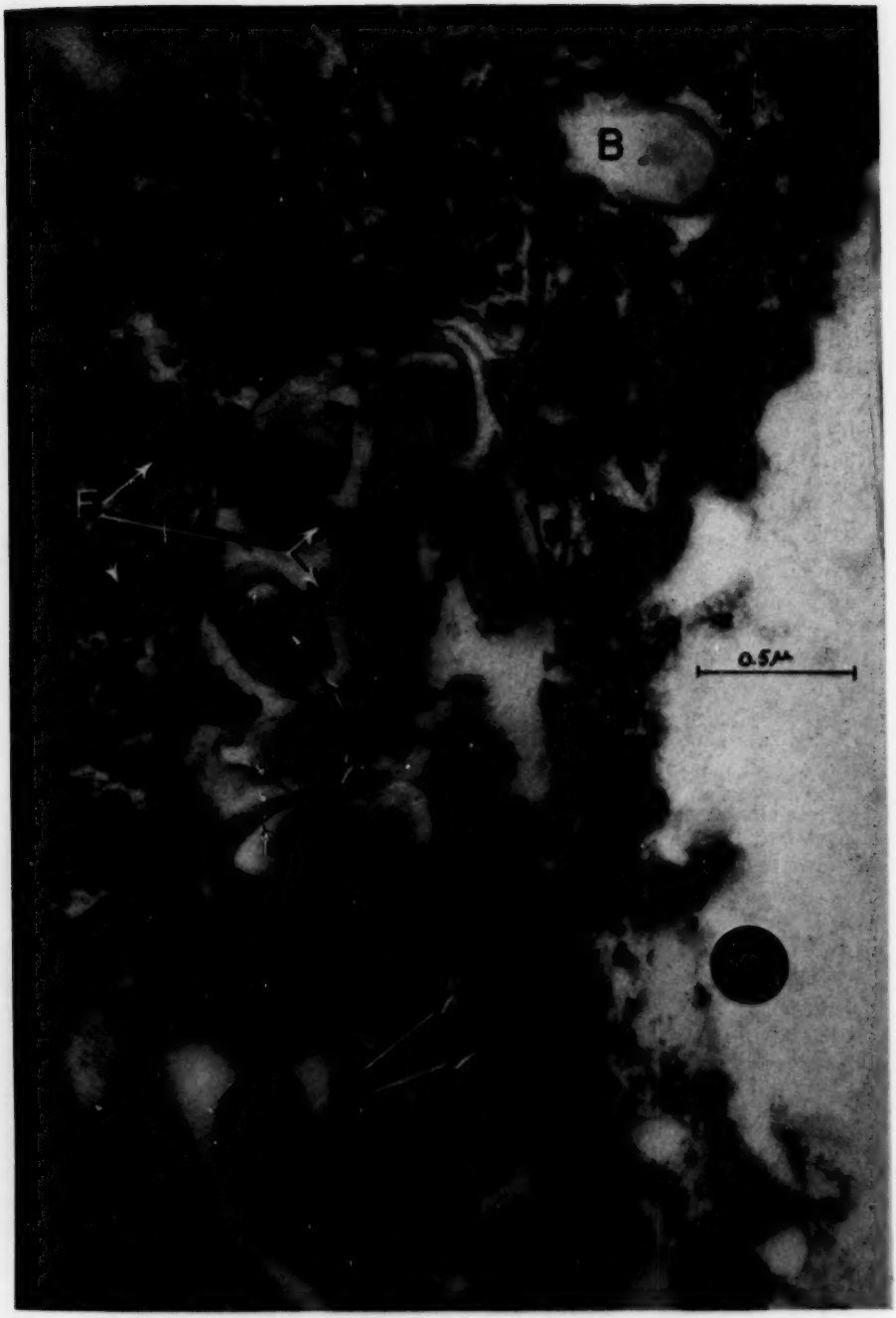

FIG. 7.—Section of soft carious dentin decalcified and stained with phosphotungstic acid. Numerous micro-organisms are seen within the matrix (*B*). The collagenous fibrils (*F*), most of which are cut in cross-section, appear to be structurally intact. A fine fibrillar or granular substance (*S*) is seen between collagenous fibrils. (Mag. approx. 45,000.)

The matrix was devoid of crystallites in a few small areas, but these areas did not follow any detectable pattern of distribution.

In homogenate preparations (Fig. 11) the crystallites were often found to lie with their long axes parallel to those of the collagenous fibrils. Unfortunately, it was not possible to define the precise relationship between fibril and crystallite; it could not be ascertained whether crystallites were within the fibrils, attached to them, or free in the ground substance; nor could a relationship be observed between the position of crystallites and the periodic structure of the fibrils.

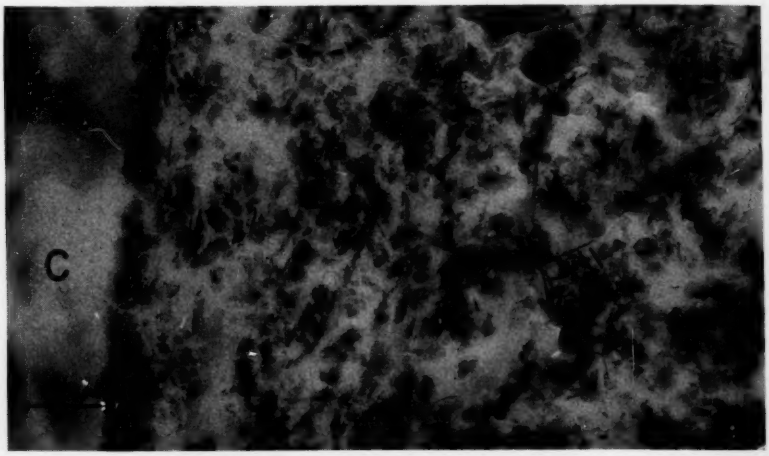

FIG. 8.—Undecalcified soft carious dentin. This section was cut from methacrylate-imbedded material. Crystallites seen in edge view present thin, dark profiles; those seen in broad surface view are wider and lighter. At left is the lumen of a dentinal canal (*C*). (Mag. approx. 110,000.)

FIG. 9.—Undecalcified soft carious dentin showing the somewhat crumbled edge of a section taken from unimbedded material. The crystallites appear thin and dark in edge view and broad and pale in broad surface view. (Mag. approx. 100,000.)

Between the lumen of the dentinal canals and the typical intercanalicular matrix a heavily calcified layer of material was frequently interposed. This layer was discontinuous. In some places the discontinuity was obviously due to fragmentation; in other places there was nothing to indicate that absence of the heavily calcified layer represented a technical artifact. Figures 5, 8, and 10 show considerable variation in thickness of the hypercalcified layer, and it will be observed to be trilaminar in the thickest example, with the largest and most loosely packed crystallites in the middle (Fig. 10). These latter-named crystallites appear to be platelets like those of the intercanalicular matrix; the others are too tightly packed to permit accurate morphologic observations. The crystallites of the hypermineralized zone, like those of the intercanalicular matrix, gave diffraction patterns typical of apatite. No large, free calcified bodies were found within the canals.

Organic phase.—As stated previously, treatment of a section of undecalcified carious dentin with phosphotungstic acid solution simultaneously decalcified the section and sharply stained the collagenous fibrils of the intercanalicular matrix, the bacterial

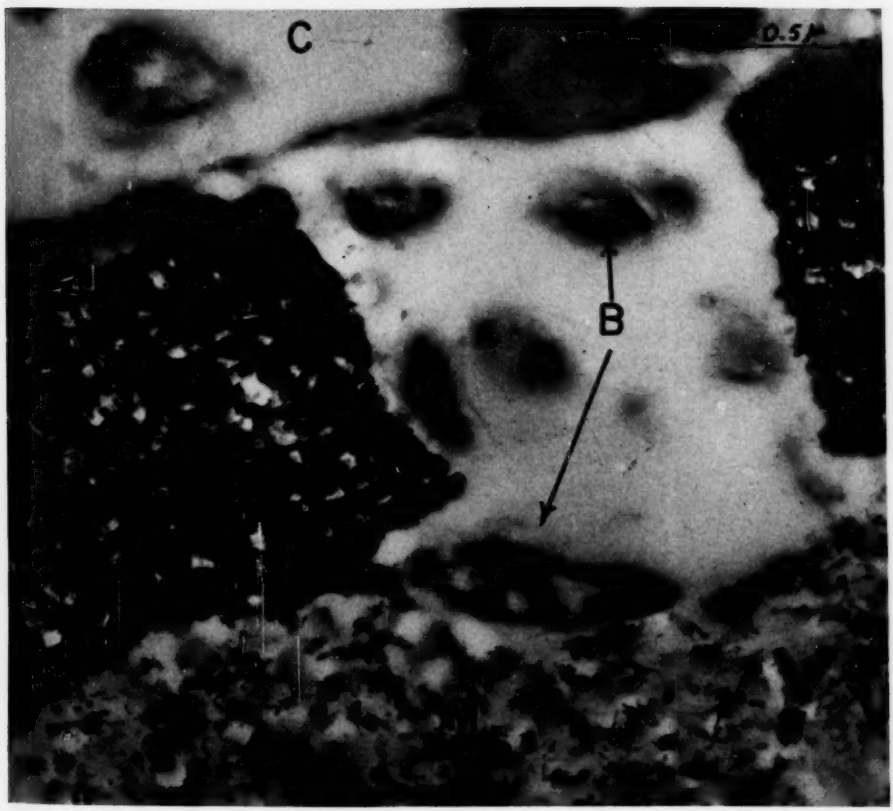

FIG. 10.—Undecalcified section of soft carious dentin showing a thick, densely calcified layer interposed between the lumen of the canal (*C*) and the intercanalicular matrix (*M*). In this instance the layer appears trilaminar with thin, dense inner and outer layers and a thick, but less dense, middle layer. Bacteria (*B*) are found in the break of the lining. (Mag. approx. 41,000.)

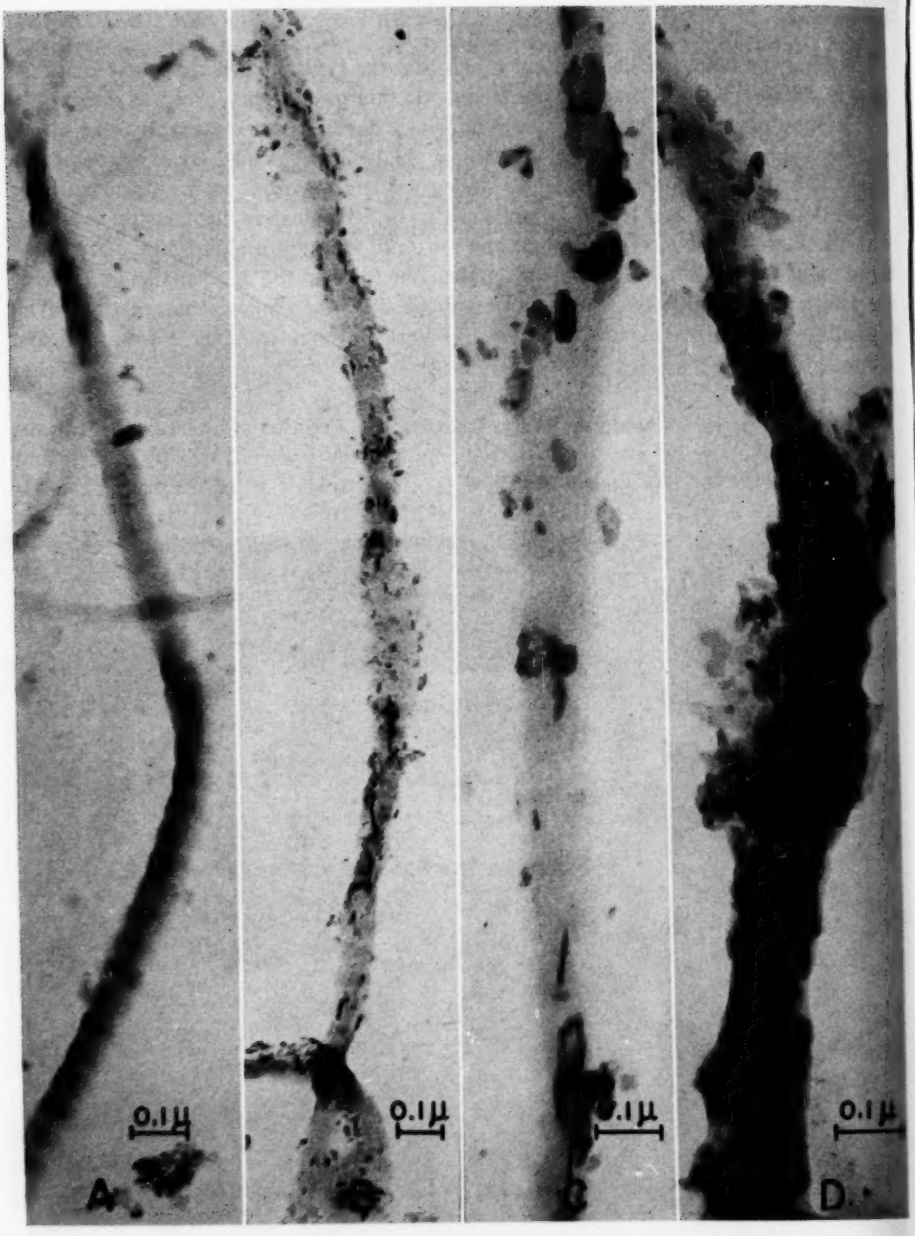

FIG. 11.—Isolated collagenous fibrils from carious dentin (homogenate preparation). *A*, collagenous fibril showing banding and devoid of crystallites (approx. 80,000); *B*, collagenous fibril with slight indication of banding, with the longer crystallites tending to be oriented with their long axes parallel to the long axis of the fibril (approx. 65,000); *C*, collagenous fibril with very faint indication of banding in some areas. A few crystallites in both broad surface view and narrow profile view are seen associated with the fibril (approx. 95,000); *D*, heavily mineralized fibril with the same preferred orientation of crystallites. (Mag. approx. 110,000.)

membranes, and perhaps to some extent the amorphous ground substance of the matrix. The collagenous fibrils, unlike the crystallites, were usually rather densely packed, often tending to run a parallel course (Figs. 3, 12) but sometimes interlaced to form a feltwork (Figs. 7, 13). In a few places, where decay was probably most advanced, the fibrils were more sparsely scattered (Fig. 12). Some fibrils showed typical cross-striations (Fig. 3), while others did not. Between the fibrils was a possibly granular, possibly fibrous, fine-textured material that may have represented ground substance, organic material of salivary origin, or possibly a product of collagen breakdown.

In homogenate preparations (Fig. 11) some of the collagenous fibrils were seen to have typical transverse bandings, while others appeared to be homogeneous. Apatite

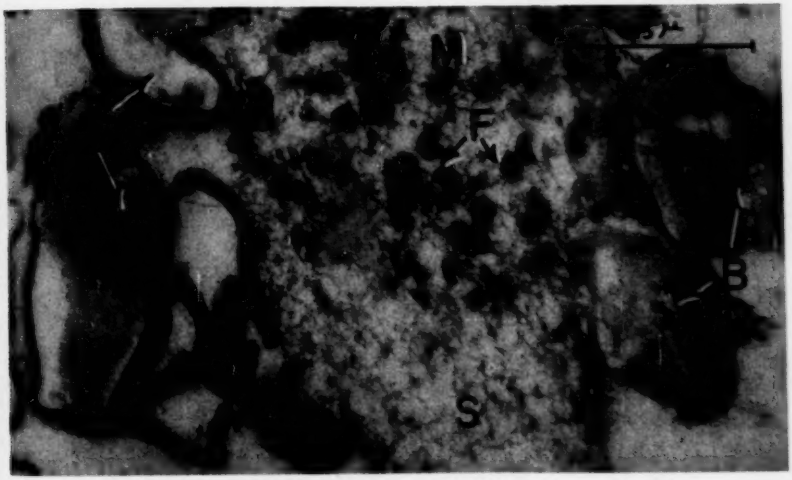

FIG. 12.—Section of soft carious dentin decalcified and stained with phosphotungstic acid. Microorganisms (*B*) are found in close proximity of the matrix (*M*), which contains only a few collagenous fibrils (*F*) seen in cross-section. The matrix in the lower third of the field is devoid of collagenous fibrils (*S*). This material may represent breakdown products of collagen, an organic substance from saliva, or the residue of the densely calcified lining material (*P*, Fig. 13). (Mag. approx. 53,000.)

crystals were scattered along the course of both types of fibril. An amorphous substance was found inclosing groups of fibrils.

In sections decalcified with phosphotungstic acid the residue of the hypercalcified layer lining the dentinal canals appeared to be finely granular in texture and was devoid of collagenous fibrils (Fig. 13).

DISCUSSION

One of the outstanding peculiarities of the carious dentin described in this study is the pattern of distribution of mineral matter. The heavily mineralized layer lining the dentinal canals stood in strong contrast to the extensively, though incompletely, demineralized intercanalicular matrix. Of further interest was the fact that many of the crystallites of both regions of calcified tissue were relatively large, comparing favorably with crystallite dimensions that have been seen in normal dentin.[7] One possible explana-

tion for the presence of these intact, or relatively intact, crystallites is that they were selectively spared when the main mass of inorganic material of the dentin underwent dissolution. If such were the case, it might possibly be due to the presence of fluoride within the crystallites.[8] This notion seems to be more readily applicable to the pericanalicular lining than to crystallites of the intercanalicular matrix. Fluoride appears to deposit preferentially near the surface at which the ions come into contact with tooth substance, i.e., at the outer surface of enamel,[9] and at the pulpal surface of dentin.[10] Therefore, it would not be remarkable if fluoride ions within dentinal canals deposited

FIG. 13.—Section of soft carious dentin decalcified and stained with phosphotungstic acid. In the lower part of the field, collagenous fibrils of the intercanalicular matrix are seen in both transverse and longitudinal section (*F*). In the lumen of a canal (*above*) are seen parts of some micro-organisms (*B*) somewhat shrunken away from a more or less homogeneous coagulum (*G*). Between lumen and matrix is the non-collagenous, fine-textured residue of the densely calcified pericanalicular lining material (*P*). (Mag. approx. 54,000.)

preferentially within those crystallites nearest to the luminal surface. Another possible explanation for the presence of large crystallites in this markedly demineralized tissue is that they were not survivors of the demineralization process but represented a product of recrystallization. Isotope studies have shown that an ionic-exchange phenomenon occurs by which salivary calcium and phosphate ions are deposited in sound dental tissues.[11] The materials for possible recrystallization in carious dentin might thus come from saliva. It is further possible that neutralization of acids produced within carious cavities from ingested carbohydrates might also permit recrystallization of ions that had been recently dissolved from dentinal tissue. In view of recent findings in studies

of "defect apatites"[12, 13] the possibility might also be considered that the remaining crystallites were more perfectly formed than those which had dissolved.

The heavily calcified layer lining canals presents a morphologic problem of interpretation, in addition to the chemical questions raised above. Whether it represented the intact persistence of a structural entity that pre-existed in normal dentin or was a secondary formation incident to the carious process cannot be answered at present. This layer was clearly identifiable in decalcified, stained sections; its matrix differed from the intercanalicular matrix, in that it contained no identifiable collagenous fibrils; but it was not markedly different in texture from the interfibrillar material of the intercanalicular matrix, and no sharp line of demarcation was apparent between the two zones of tissue.

All stained sections and homogenate preparations of soft carious dentin contained an abundance of collagenous fibrils. The amount of collagen relative to the volume of dentinal matrix was, in our judgment, just as copious (cf. Fig. 4) as in normal dentin. Only occasionally were areas characterized by a sparseness of collagenous fibrils observed (Fig. 12), and then there was difficulty in determining to what extent we were dealing with the non-collagenous matrix of the pericanalicular lining. Some question exists as to the state of the collagenous fibrils in soft carious dentin. As stated above, some fibrils exhibited typical cross-striations, while others did not. This difference could not be related to the nearness of a fibril to bacteria; Figure 3 shows striated fibrils in a spicule of matrix at the surface of the lesion. However, in other places, fibrils that were some distance from bacteria showed no striations. It is not possible to say at present whether the failure of some collagenous fibrils to show striations was due to the decay process or to some imperfection in the technical methods at our disposal. In general, it can be said that the collagenous moiety of the matrix was reasonably intact except in places where the matrix had been completely destroyed and replaced by bacteria.

In view of the rather uniform distribution of collagenous fibrils and of apatite crystallites in the intercanalicular matrix and the absence of transitional zones in soft carious dentin, it appears that, apart from the fact of an obvious bacterial invasion, the carious destruction of dentinal matrix can be grossly divided into two stages: (1) a wave of demineralization that dissolves most of the crystallites but leaves the collagenous matrix relatively intact and (2) a simultaneous, or almost simultaneous, destruction of the collagenous matrix and remaining crystallites.

SUMMARY

An electron-microscopic study was conducted on soft carious human dentin fixed in buffered osmic acid, 10 per cent neutral formalin, or 70 per cent alcohol and imbedded in butyl methacrylate. Some material was studied without fixation and imbedding. For observations on the inorganic phase of the tissue and collagen-crystallite relationship, unstained sections and tissue homogenates were employed. For study of the organic phase, sections were stained in phosphotungstic acid. It was found that even in advanced dentinal caries the typical morphologic pattern of dentin is recognizable with massive bacterial colonies covering the surface of the lesion and extending into the dentinal canals and intercanalicular matrix. A general and possibly selective demineralization of the inorganic phase appeared to be the first phase in the carious process, leaving a sparse distribution of crystallites in the intercanalicular matrix and a distinct, heavily

mineralized pericanalicular layer. The finding of an abundance of apparently normal collagenous fibrils indicated that collagen breakdown followed a general tissue demineralization. The final stage in tissue destruction apparently involves the more or less simultaneous dissolution of remaining crystallites and depolymerization of the collagen fibrils.

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Effects of Heat and Sonic Oscillation on Cytochrome c

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The development and utilization of high-energy tools in dentistry has increased the need for knowledge of the mechanisms of injury to living tissues from such sources. Although histologic methods have been used to evaluate tissue changes,[1–6] it would appear that few studies have been made of the biochemical or metabolic aspects of these problems. Since the deleterious effects of high-energy tools are mediated primarily via heat and vibrations, it was thought advisable, preliminary to more detailed studies, to compare the effects of heat and of sonic oscillations on a relatively pure oxidative enzyme, cytochrome *c*, which is found in all mammalian tissues that utilize oxygen.[7–11] The cytochrome *c* content of tissues directly parallels and is proportional to their aerobic oxidative metabolic capacities.[12]

It is very pertinent to the above considerations that, while the present studies were in progress, Belding, Fisher, and Braley[13] reported that exposure of bovine dental pulps to sonic oscillation inhibited oxygen utilization of the tissue. The present studies, then, assume added significance in that they shed light upon possible mechanisms of inactivation of a critical component of the chain of terminal respiratory enzymes which make it possible for mammalian tissues to utilize molecular oxygen.

In the present study, solutions of cytochrome *c* were exposed to a temperature of 100° C. for periods up to 2 hours and, in separate experiments, to sonic oscillation for periods up to 5 hours. Changes in the enzyme were followed by (1) spectrophotometric determination of light absorption and (2) manometric determination of catalytic activity. In addition, in the sonic experiments, determinations were made of migration patterns in a paper electrophoretic field, of sedimentation patterns in the ultracentrifuge, and of free amino acids by paper chromatography.

It is emphasized that treatment of the enzyme was deliberately drastic and exceeded physiologic limits in the hope of producing maximal molecular changes.

EXPERIMENTAL METHODS

Beef-heart cytochrome *c* (Sigma), of 65–70 per cent purity, was used. The cytochrome *c* was dissolved in 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, or in distilled water. The range of final concentrations employed in most experiments was between 0.024 and 0.380 mg/ml. When exposed to heat or sonic oscillation, the compound was in the oxidized form.

In the heating experiments, cytochrome *c* was dissolved in buffer or in distilled water. A thin-walled, ground-glass-stoppered test tube containing the solution was

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placed in a boiling-water bath, and aliquots were removed for analysis at 10- or 15-minute intervals following attainment of boiling temperature. Exposure to sonic oscillation was accomplished by the use of a sonic oscillator* operating at 9-kc capacity (see Fig. 1). The stainless-steel cup which held the solutions during sonication was cooled by circulating ice water. Non-oscillated cytochrome *c* control solutions, in vitro or in stainless steel containers, were kept in a cold room at 4° C., and aliquots of these were removed for analysis concomitantly with the sonicated or heated samples.

Continuous tracings of absorption spectra were made with a recording spectrophotometer.† The absorption spectra of the control solutions and of the treated solution

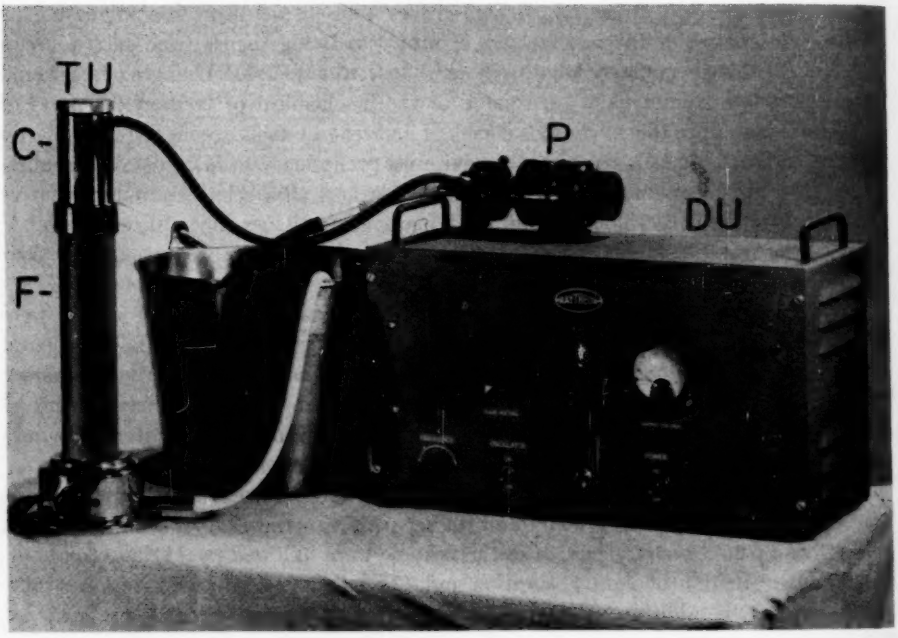

FIG. 1.—Photograph of sonication apparatus ready for use. *DU*, driver unit containing power supply and oscillator; *TU*, treatment unit consisting of *C*, a stainless-steel sample holding cup, from the base of which a laminated nickel rod (not visible) extends into *F*, the field coil component. *P* is a pump which circulates ice water through the treatment unit assembly while it is in operation. The stainless-steel bucket, *B*, serves as reservoir for the circulating water. The driver unit and treatment unit together comprise the Raytheon 50-watt, 9-kc Magnetostriction Oscillator, model S-102-A. In experimental use, the unit was operated at full power, i.e., 50 watts, 9 kc.

were determined in both oxidized and reduced forms. Sodium hydrosulfite was used as the reducing agent. The catalytic activity of cytochrome *c* was determined by a modification of a standard cytochrome oxidase assay method.[12, 14] The modification consisted of lowering the concentration of cytochrome *c* in the assay, in order to make cytochrome *c*, and not the oxidase, rate-limiting. Ultracentrifugal analyses were made in an ultracentrifuge‡ according to the method of Schachman.[15] In the paper electro-

* Raytheon, model S-102 A.

† Process and Instruments Recording Spectrophotometer, model RS 3.

‡ Spinco Ultra-centrifuge, model E.

phoresis experiments, the electrophoretic apparatus* was used as described by Durrum.[16] Paper chromatographic analyses for free amino acids were made, using the butanol, acetic acid, water (4:1:1) system of Block, LeStrange, and Zweig.[17] With the exception of the manometric determinations of catalytic activity, in all experiments following exposure to heat and to sonic oscillation, both control and experimental solutions were subjected to centrifugation at 25,000 × *g*. for 1 hours at 4° C. prior to measurement of the various properties. This was necessary to remove particulate matter which formed as a result of heat treatment or of sonic oscillation, since such material would interfere with light absorption, sedimentation patterns, paper electrophoresis, and chromatography.

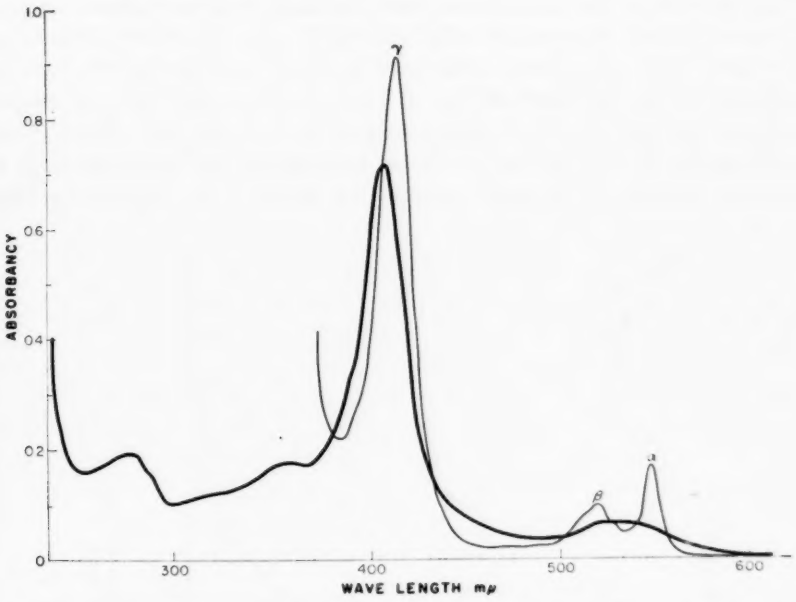

FIG. 2.—Reference absorption spectra of reduced (*light line*) and oxidized (*heavy line*) cytochrome *c*. The characteristic absorption spectrum of the compound is that of its reduced form in which three absorption maxima are present. Starting from the long-wave-length region, the absorption peaks of the reduced cytochrome are the alpha (α) at 550 mμ, beta (β) at 520 mμ, and soret (γ) at 415 mμ. In the oxidized form, the α and β peaks fuse into a broad maximum of diminished intensity. The soret peak shifts to 408 mμ and is also diminished in intensity.

RESULTS

Changes in light absorption.—Oxidized cytochrome *c* is red, while the reduced compound is pink. In Figure 2, for reference purposes, may be seen the absorption curves of reduced (*light line*) and oxidized (*heavy line*) cytochrome *c*. The characteristic absorption peaks or maxima of cytochrome *c* seen in the reduced compound are the alpha (α) peak at 550 mμ, the beta (β) peak at 520 mμ, and the gamma (γ) or soret peak at 415 mμ. The ultraviolet absorption of the reduced enzyme is not shown

* Spinco Electrophoresis Apparatus, model R.

below 400 mμ, since hydrosulfite, the reducing agent, absorbs strongly in this region and hence interferes with the determination.

In the oxidized state (i.e., Fe^{3+}) the α and β peaks disappear and are replaced by a single, broad, flat absorption hump between 520 and 540 mμ. The γ or soret absorption is shifted from 415 mμ to 408 mμ and is diminished in intensity. In the ultraviolet, a shoulder at 355 mμ is present, belonging to the porphyrin component, and also a peak at about 280 mμ, caused by aromatic amino acids in the protein moiety of the enzyme.[18]

The effects of 2 hours' exposure to 100° C. upon the absorbancy of cytochrome *c* are shown in Figures 3 and 4. In Figure 3 the absorbancies at the 408-mμ and 520–540–mμ maxima of the treated compound (oxidized form) are shown. In Figure 4, the treated material was reduced with hydrosulfite prior to determination of absorbancy values. The values shown have been corrected for base line (i.e., non-specific absorbancies). The left-hand ordinate of Figure 3 shows absorbancy of the 408-mμ peak, while the right-hand ordinate shows absorbancy of the 520–540-mμ maximum. The changes in the 350-mμ and in the 280-mμ peaks of the ultraviolet could not be quantitated in these experiments because their behavior was masked by relatively

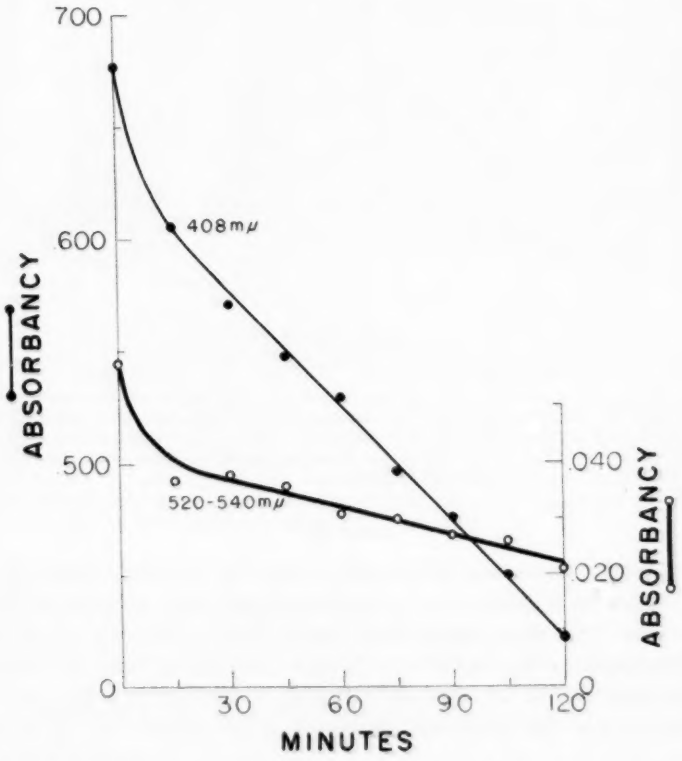

FIG. 3.—Loss in absorbancy of oxidized cytochrome *c* during 2 hours of heating at 100° C. Original concentration: 0.14 mg. cytochrome *c*/ml.

large, non-specific, base-line absorbancy changes which were equal to, or greater than, the specific absorbancy changes. It may be seen for each of the visible maxima that during the first 15 minutes there was an initial rapid fall in absorbancy, followed by a lesser, constant rate of decline during the remainder of the experiment. It is also evident that the rate of absorbancy loss was greater for the 408-mμ peak than for the 520–540-mμ maximum.

In Figure 4 are shown the absorbancy changes in the heated cytochrome reduced spectrum. The left-hand ordinate shows absorbancy values of the γ peak; the right-hand ordinate shows absorbancy values for the α and β peaks. It may be seen again that rapid losses in absorbancies occurred in the first 15 minutes, followed by somewhat slower, constant rates of decline during the remainder of the experiment. The soret of γ absorption at 415 mμ exhibited the most rapid rate of change (as was the case with its counterpart at 408 mμ in the oxidized spectrum). The slopes of the curves depicting the changes in the α and β peaks are approximately equal to one another, and also with that of the 520–540-mμ oxidized maximum. This is to be expected, since these absorbancies derive from the same source within the molecule.[18]

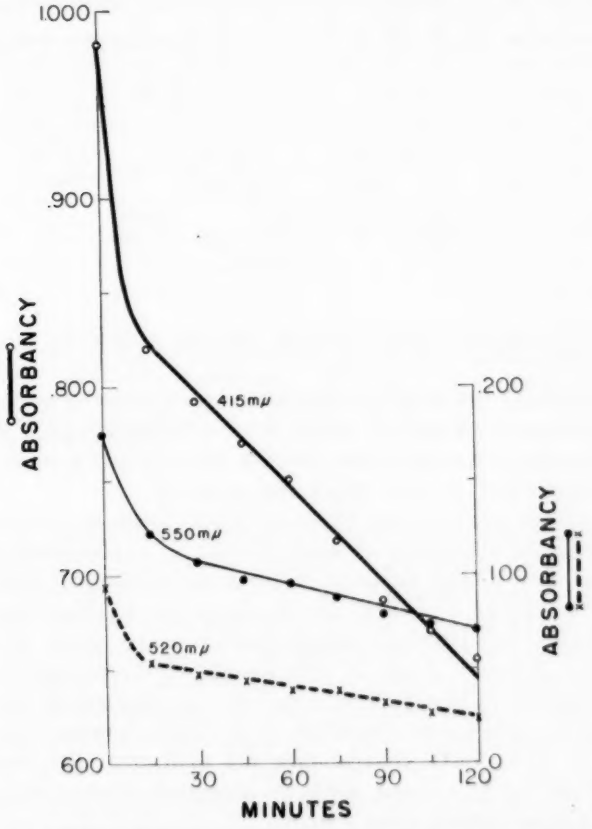

FIG. 4.—Loss in absorbancy of cytochrome *c* during 2 hours of heating at 100° C. Reduced following heating. Original concentration: 0.14 mg. cytochrome *c*/ml.

Although the *absolute* loss in absorbancy was greatest for the γ peak, on a percentage basis the peak lost only 33 per cent of its beginning absorbancy, while the α and β peaks lost 59 and 76 per cent, respectively, of their beginning absorbancies.

Sonic oscillation produced a decrease in absorption maxima, as did heat treatment. Although changes produced by sonic oscillation were followed at hourly intervals, the precision of results at such intervals was poor, i.e., less than ±10 per cent when experiments were repeated. This may have been caused, in part, by uncontrollable fluctuations in the house-line current from which the instrument operated. It was found, however, that, while hourly values were not reproducible, over-all changes per 5-hour interval were reproducible to ± 10 per cent. For this reason, in the sonic oscillation experiments, light absorption changes will be presented upon a 5-hour basis.

In Table 1, *a*, typical data are given for changes in the absorbancy of oxidized cytochrome *c* following 5 hours of sonic oscillation. Only the 408-mμ and the 520–540-mμ peaks could be quantitated for reasons given previously. The 408-mμ peak

TABLE 1

LOSS IN ABSORBANCY OF CYTOCHROME *c* AFTER 5 HOURS OF SONIC OSCILLATION

Time (Hours)	*a*) Oxidized Spectrum Maxima (mμ)				*b*) Reduced Spectrum Maxima (mμ)					
	408		520–540		415 (γ)		520 (β)		550 (α)	
	A*	Per Cent Change	A	Per Cent Change	A	Per Cent Change	A	Per Cent Change	A	Per Cent Change
0.....	0.730	.....	0.062	.....	0.998	.....	0.097	.....	0.178	.....
5.....	0.353	−52	0.033	−47	0.499	−50	0.026	−73	0.070	−61

* A = absorbancy.

lost 52 per cent and the 520–540-mμ hump 47 per cent of their beginning absorbancy values.

In Table 1, *b*, typical data are given for the changes in absorbancy of the α, β, and γ peaks of cytochrome *c* following 5 hours of sonic oscillation (and subsequent reduction by hydrosulfite). It may be seen that for the α, β, and γ peaks, respectively, losses in absorbancy of 61, 73 and 50 per cent occurred.

Changes in catalytic activity.—In Figure 5 typical losses in catalytic activity of solutions of cytochrome *c* following exposure to 100° C. or sonication are shown. In the assay method used, a fixed amount of cytochrome oxidase mediated oxygen consumption. The cytochrome *c* required for the assay was supplied from the treated solutions and was the rate-limiting component of the assay system. It may be seen from the curves in Figure 5, *A* and *B*, that the effects of heat treatment and sonic oscillation were similar, in that both produced the same pattern of loss of catalytic power of the exposed cytochrome *c* solutions. It is evident, however, that exposure to 100° C. produced more drastic changes than did sonication. The heated solutions lost all catalytic activity in an hour, while the sonicated solutions still possessed 54 per cent of the original activity after 5 hours.

Paper electrophoresis, paper chromatography, and ultracentrifugal analyses.—Pre-

liminary experiments were performed using the above techniques, in order to determine whether or not detectable changes in size, charge, or hydration of cytochrome *c* occurred following sonic oscillation. Also the chromatographic analyses would show whether or not free amino acids or peptides were cleaved from the molecule. There were no significant differences between oscillated and non-oscillated control samples, using any of the above techniques. It is obvious, therefore, that such changes in size, charge, and hydration of cytochrome *c* as did occur were associated with loss of solubility of the material and formation of denatured, precipitated enzyme.

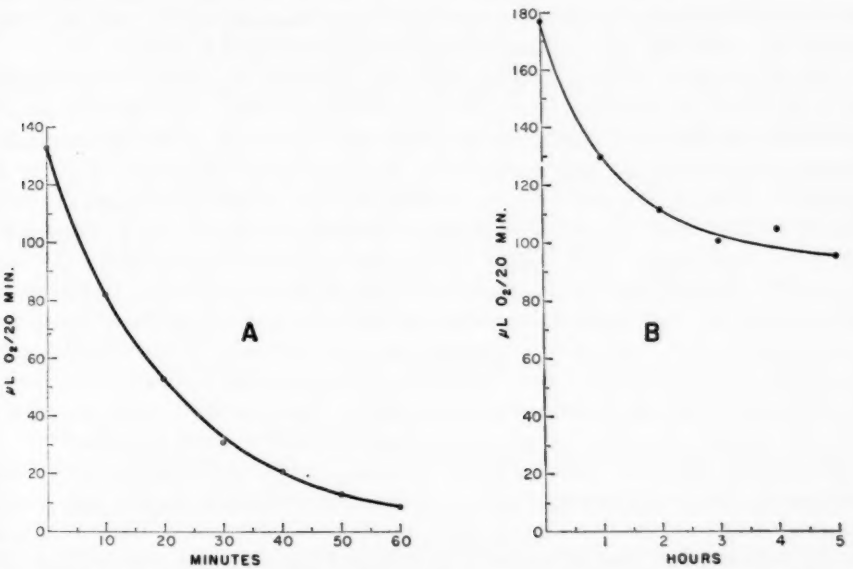

FIG. 5.—Loss in catalytic activity of cytochrome *c* as a result of heat treatment (*A*) and following sonic oscillation (*B*). Manometric system: ***main body of flask,*** 0.25 ml. semi-carbazide-HCl, pH 7.0; 0.25 ml H_2O; 1 ml. beef heart cytochrome oxidase preparation, pH 7.0; 1.0 ml. cytochrome *c* (Sigma), pH 7.0, original concentration 0.65 mg/ml; ***side arm,*** 0.3 ml. hydroquinone (= 3 mg.) in H_2O; ***center well,*** 0.2 ml. of 10 per cent NaOH.

DISCUSSION

It is generally considered that the effects of ultrasound are mediated via (1) thermal, (2) mechanical, and (3) chemical forces.[19, 20] The thermal effects result from the absorption of sound energy in the form of heat. Mechanical effects result from cavitation, in which bubbles of vapor appear in the medium, and also from deformation of the medium by the sound waves. The chemical effects are primarily oxidative and degradative in nature and are thought to be closely linked with the cavitation phenomenon. It is difficult and, indeed, often impossible to assign relative quantitative influences to each of the above factors in a given experimental system. For critical discussions of the factors involved, the reader is referred to the capable treatments of the theoretical and practical aspects of these problems contained in references 19–22.

Turning to the experiments reported in this paper, it is evident that the effects of sonic oscillation and heating upon solutions of cytochrome *c* were, with one exception,

similar qualitatively and differed only in degree. Concomitant with both procedures, there was a loss in solubility and denaturation of the enzyme, as evidenced by the decreases in absorbancy of the various maxima. However, *only* sonic oscillation produced a very fine black sediment which did not give the xanthoproteic reaction for protein with nitric acid; nor did it form a hemochromogen with pyridine. The possibility exists that this sediment arose from carbohydrate moieties within the molecule. The spectrophotometric and manometric data suggest that mechanisms of damage as a result of exposure to sonic oscillation and to heat were similar at molecular levels and that denaturation of the enzyme occurred. Under the conditions of these experiments, heat treatment produced a more rapid denaturation, but it must be remembered that relatively low oscillatory frequencies were used, i.e., 9 kc.

The experiments reported in this paper are important in considering mechanisms of pulp injury at molecular levels. Recently Belding, Fisher, and Braley[13] reported that sonic oscillation of bovine dental pulps was capable of producing decreases in oxygen consumption by pulp tissue slices. The pulps were exposed to 9–10 kc frequencies, using an instrument similar to ours, for 3, 5, 10 and 15 minutes. Q_{O2} values during the first hour of in vitro respiration were depressed by 11.5, 2.8, 1.5, and 32.6 per cent, respectively. It is evident in the abstract report[13] from which the above data were obtained that the metabolic response of the pulp to sonic oscillation was characterized by a high variability, which we have also noted with the relatively pure cytochrome *c*. This variability in response to sonic oscillation is less troublesome to comprehend if one considers that the cavitation phenomenon responsible for most of the chemical effects of the procedure is in itself a highly variable event in regard to size and number of cavitation spheres produced per unit of space and time.[19, 20]

We cannot apply our results directly to intact tissues which contain many other components than cytochrome *c* and in which bioarchitectural factors not possibly involved in our studies are also of great metabolic significance.[20] There can be no doubt, however, that heat denaturation of critical respiratory and other enzyme components of living tissues should be considered as a mechanism involved in pulp injury from high-energy tools.

SUMMARY

A study was made of the effects of exposure of solutions of cytochrome *c* to 100° C. for 2 hours and to sonic oscillation (9 kc) for 5 hours. The primary changes revealed by spectrophotometric and manometric determinations in both types of experiment were an irreversible loss of solubility, denaturation of the enzyme, and loss of catalytic activity. In addition, sonic oscillation produced a very fine black sediment, not seen as a result of heat treatment.

We are indebted to Mr. Daniel O'Connell, of the Basic Medical Research Laboratory of this hospital, for performance of the electrophoresis and ultracentrifuge analyses, and to Mr. Herbert Zipper, of the Radioisotope Laboratory, for performance of the paper chromatographic analyses.

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Silicate Cements and Acid Production

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The acidic nature of silicate cement has been imputed in the pulpal degenerations that occur beneath unlined silicate cement restorations. The statement, "a low pH" has been used to indicate that silicate cement has an acidic nature. But pH, a measure of the hydrogen-ion concentration in solutions, is not suitable for directly describing some property of solids.

Leaching solutions have therefore been used to determine whether the set cement when exposed to a liquid will produce acid, but again the use of pH measurements has been misleading. For example, when distilled water is exposed to air, the solution of carbon dioxide increases the hydrogen-ion concentration, and the pH may fall to 5.0; Paffenbarger, Schoonover, and Souder[1] recognized this in 1938, but advertisers and dentists still talk about silicate cement as having a pH of 5.0, because some of the papers[2–6] reported such a pH when distilled water was used as a leach.

To obtain more meaningful results, the amount of acid produced by solution of the silicate cement in some leach must be measured by titration with alkali. In solutions of metallic oxide complexes the hydrogen-ion concentration is dependent on the concentration of the various salts or complex ions formed; the addition of acid or alkali to such solutions produces a variety of pH changes due to the changing solubilities and concentrations of the dissolved salts.

Some information about the reaction of silicate cement to leaches was obtained when wafers of silicate cement were immersed in three different leaches. Because the concentration of the acid produced by the silicate cement is affected by the salts in the surrounding solution and in order to relate the information to the oral use of silicate cement, physiologic fluids were used, and the results obtained were compared with those using distilled water as a leach.

MATERIALS AND METHODS

A standardizing mixing and forming procedure was used to produce cylindrical wafers of silicate cement. The powder* was measured (1.40 gm.) on a chemical balance (± 0.005 gm.) and the liquid (0.4 ml.) in a tuberculin syringe (± 0.05 ml.). After mixing for 1 minute on a chilled glass slab, the cement was pressed out between glass slides to produce a wafer 2.5 mm. in diameter. After 30–60 minutes, each wafer was placed in a Petri dish and covered by 10 ml. of leaching solution. The lid was placed on the dish and the dish left undisturbed until the amount of acid was estimated by titration.

This work was done at the University of Otago Dental School in Dunedin, New Zealand.
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* S. S. White Filling Porcelain Improved; batch number not available.

A randomization procedure was used to allocate disks to dishes, so as to avoid bias when different solutions were used; the time from setting to immersion—that is, the degree of set—affected the amount of acid produced.

The solutions used as leaches were distilled water (pH 5.5–6.0), Ringer's solution (pH 7.0), and Ringer's solution with buffer (pH 7.0, buffered from pH 6.0 to 7.0). The solutions were supplied by the Department of Biochemistry, University of Otago, and were standard physiologic fluids. The solutions were pipetted from refrigerated stock, which was renewed weekly.

Estimations.—After 48 hours, 5 ml. of leach were withdrawn (micropipette graduated in 0.01 ml.) and titrated against approximately N/100 sodium hydroxide from a microburette (graduated in 0.01 ml.), using phenolphthalein as an indicator. Before and after each series of six titrations the stock alkali was titrated against N/100 standard acid solution (potassium hydrogen phthalate), and thus all values were obtained in terms of N/100 acid.

The variation in the amount of alkali required to bring the Ringer's and buffered Ringer's solution to pH 8.4 (phenolphthalein) indicated that the stock of these solutions could not be kept in a constant state and that, as the estimations were done over several weeks, comparisons between all sets of values for the acid produced by the silicate cement would not be valid. Hence comparisons were made only with estimations from specimens in stock solutions of similar age and composition.

The results were compared by the analysis of variance.

In all but Table 1, no account was taken of the alkali required by the solution itself to bring the pH to 8.4. Thus the rows can be used only for comparisons within each table: the addition or subtraction of a constant amount (representing the alkali required for the solution alone) does not alter the analysis of variance. The confidence interval was based upon the Student *t*-test.

DISCUSSION AND RESULTS

This simple experiment serves only as a guide for thought and as a basis for a hypothesis; no notions of a quantitative value for the acid production of silicate cement restorations are entertained.

Table 1, *a*, illustrates a well-known chemical phenomena,* that is, in the presence of a buffer, the addition of acid or alkali alters the pH but little until the buffer is "used up." To change the pH from 5.0 to 8.4 in the sample of distilled water required 0.03 ml. of alkali; in the sample of Ringer's solution with the buffer added, 1.71 ml. of alkali were needed to change the pH from 7.0 to 8.4. The importance of the buffering is shown when the silicate cement wafer is immersed in the various solutions. As the constituents of the silicate cement dissolve to produce an acid state, the pH of the surrounding liquid is altered; the solution of the silicate in turn is related to the pH of the surrounding liquid and hence that solution which changes least in pH during leaching of the cement shows the greatest quantity of acid present at the conclusion of the leaching. The presence of various salts in the Ringer's solution was apparently enough to increase the solution of silicate cement.

* While this statement may seem trivial, many users of dental materials are unaware of the effect of buffers upon the solution of solids.

In Table 2 the effects of halving the surface area are shown, without altering the volume of material available for leaching. This table should indicate how small the amount of acid produced on the pupal aspect of a silicate restoration in the mouth must be. In this experiment an area of silicate about 18–20 cm.^{2} produced about 4–5 ml. of N/100 acid under conditions more favorable for leaching than those within a tooth.

In another series (Table 3), a slice of tooth—a horizontal section of a molar crown which had been stored in water after extraction—was placed with the silicate cement wafer in the leaching solution. The leached acid combined directly either with the tooth material or with dissolved tooth material. Thus the acid, if leached out of a silicate cement in a tooth, might well be neutralized by the tooth material.

Some silicate wafers were varnished with a simple dental varnish that was supposed to protect the cement from water. The varnish was appplied liberally to the wafer, and a complete covering was most likely attained. Varnish in the leaching solution alone

TABLE 1*

SILICATE CEMENT IN LEACHING SOLUTIONS

	a) Alkali in Milliliters Required by Solutions to pH 8.4						
D.....	0.04	0.04	0.03	0.03	0.03	0.03	Mean=0.03
R.....	0.18	0.16	0.20	0.20	0.19	0.17	Mean=0.18
RB.....	1.69	1.76	1.69	1.71	1.76	1.69	Mean=1.71
	b) Acid in Milliliters Due to Wafer of Silicate Cement						
DS.....	4.64	2.46	4.31	3.48	2.28	2.48	Mean=3.44
RS.....	3.42	3.55	3.36	3.00	3.36	2.54	Mean=3.21
RBS.....	5.87	5.99	5.60	5.90	6.17	5.37	Mean=5.82

* D, distilled water; R, Ringer's solution; RB, Ringer's solution with buffer; S, presence of silicate wafer.
Analysis of variance for *b*: $F = 12.518/0.374 = 30^{**}$ at 2, 15 d.f.
Significant difference at 1 per cent level and confidence limit for comparison of means of 0.10 at 5 per cent level.

TABLE 2

EFFECT OF SURFACE AREA
(Acid Produced in Milliliters from Specimens of Differing Surface Area but Same Volume in Ringer's Solution)

RS.....	3.60	3.73	3.54	3.18	3.54	2.72	Mean=3.39
RSH*.....	3.02	3.22	2.78	3.20	2.45	2.98	Mean=2.94

* H indicates half-surface area.
Analysis of variance: $F = 0.590/0.112 = 5.28^{*}$ at 1, 10 d.f. Significant difference at 5 per cent level.

TABLE 3

EFFECT OF TOOTH MATERIAL

BRS.....	7.58	7.70	7.51	7.61	7.88	7.08	Mean=7.53
BRST*.....	3.83	4.58	4.61	4.97	7.52	6.32	Mean=5.31

* T indicates presence of wafer of enamel and dentin.
Analysis of variance: $F = 15.253/0.959 = 15.90^{**}$ at 1, 10 d.f. Significant difference at 1 per cent level.

did not contribute any acid. Some solution of the silicate took place through the varnish and continued for at least a week.

For comparison, similar wafers of the zinc phosphate cement* were prepared in the routine manner described and immersed in the various solutions (see Table 4). However, the nature of the material differed, in that a zinc phosphate cement wafer during its setting is covered with a highly acid solution of zinc phosphate from which the zinc phosphate is crystallizing; the time of immersion markedly affected the acid "produced." In the silicate cement series, a variation of about 10 per cent was noticed and mainly attributed to differences in the length of time from mixing to immersion. By

TABLE 4

SILICATE WAFERS VARNISHED BEFORE IMMERSION IN RINGER'S SOLUTION WITH BUFFER; ACID PRODUCED, IN MILLILITERS

At 24 hours.....	0.79	1.41	2.04	Mean=1.41
At 1 week.....	0.10	0.17	0.21	Mean=0.16

ZINC PHOSPHATE CEMENT IN RINGER'S SOLUTION WITH BUFFER; ACID PRODUCED, IN MILLILITERS

At 24 hours.....	2.17	2.01	2.17	Mean=2.12
At 1 week.....	0.24	0.31	0.05	Mean=0.20

TABLE 5

RATE OF ACID PRODUCTION*

LEACH	INTERVAL							
	15 min.	1 hr.	2 hr.	4 hr.	24 hr.	3 days	5 days	14 days
RBST.....	20.64	1.01	0.52	0.10	0.08	0.03	0.01	0
DST.....	12.64	1.00	0.46	0.17	0.07	0.02	0.01	0.01

* Rate of acid production in milliliters per hour at shown intervals; each value is a mean of three specimens reimmersed for shown period in fresh solution. Tooth material was present in both leaches.

mistake, a buffered Ringer's solution of about pH 3.5 was used on one occasion, and it was noted that zinc phsophate cement in this leach produced a small amount of acid initially, then after about 8 hours the acid content of this aberrant solution was decreased, presumably by combination with the zinc phosphate wafer. Such a mechanism may also operate in lined restorations, where the acid, if any, from the silicate cement may combine with the unchanged zinc oxide in the lining cement. Without tabulated figures or statistical support it could be said that the zinc oxide produced about one-third the amount of acid in the first 48 hours and little, if any, thereafter.

The rates of acid production by the silicate wafers in buffered Ringer's solution and in distilled water were estimated. The wafers were reimmersed in fresh solution (10 ml. each time) at the intervals shown in Table 5. The silicate in the buffered

* S. S. White Zinc Cement; 1.20 gm. of powder to 0.5 ml. of liquid.

Ringer's solution again produced the most acid, but from this table it can be seen that the major amount of acid is leached out in the first 15 minutes. The rate drops off noticeably and very little could be detected after 5 days in the buffered Ringer's solution. The acid production continued in distilled water for longer.

After immersion for 1 week, the surface texture of the silicate cement wafers differed; the surface of the silicate in the Ringer's solution with buffer was detectably rougher to the touch. Also a precipitation of salts about the silicate wafer in the buffered Ringer's solution was noticed. Therefore, either a limited amount of acid was available for leaching and was removed most rapidly by the buffered Ringer's solution, or some difference in the surface chemistry occurred when the silicate was in contact with other salts. As the leaching solution was not stirred or made to flow over the wafers, the precipitation of salts about the wafer indicated that the soluble portion of the silicate cement either combined with salts in the solution or so altered the pH in the adjacent layer about the wafer that precipitation occurred from the Ringer's solution. Perhaps, had the leach been made to flow over the surface, the acid production would have been higher. These observations show some difficulties in using leaches to estimate the acid production of silicate cements in the laboratory.

CONCLUSIONS

The acid produced by a silicate cement wafer when immersed in a leaching solution will depend on the nature of the solution. Also the quantity of leaching solution used and whether the leach is stirred or not will also affect the quantity of acid produced.

The acid produced by a silicate cement when immersed in a physiologic fluid such as Ringer's solution (with or without buffer) will be greater in quantity than when a similar wafer in a similar condition is immersed in distilled water. Leaching by these physiologic solutions resembles the oral conditions.

A decrease in the surface area of the silicate cement exposed to the leach decreases the amount of acid produced, even when the same volume of silicate is used.

The presence of tooth material in the leach with the wafer decreases the acid present in the leach; the tooth material, basic in nature, neutralizes some of the acid produced by the silicate.

The solution of acid from the silicate cement by leaches is most rapid in the first hour after mixing; thereafter it diminishes rapidly and is almost undetectable after 2 weeks.

The measurement of the pH of a leaching solution about a silicate cement sample would seem to indicate solely at what pH the solution of the silicate cement ceases (within the limits of measurement). From the literature cited[1–6] and this experiment, termination of solution would seem to occur at about pH 4.0–5.0, and in some brands about 6.0. To call this the "pH of a silicate cement" seems misleading.

This experiment indicated the small, if not minute, amount of acid that might be produced on the pulpal aspect of a silicate cement restoration in a vital tooth.

Doubt about the acid leach as the sole cause of pulpal degenerations beneath unlined silicate cement restorations was created, and a theory more in agreement with the clinical aspects of such degenerations has been discussed elsewhere.[7]

SUMMARY

The simple experiment described shows that the acid produced by silicate cements on leaching is better given qualitative and quantitative value by titration with alkali than by measurements of pH. The acid produced when the leach is a physiologic fluid is larger in quantity than when the leach is distilled water. This difference may well be due to the pH of the surrounding liquid, and it is postulated that the pH referred to usually in relation to cements is that pH at which the solution of the cement ceases.

The surface area of cement exposed to leach and the elapse of time influence the quantity of acid produced. Most acid is produced within 1 hour of mixing. The basic nature of tooth material, the small amount of acid that might be leached beneath a restoration in the mouth, and the other conclusions arrived at in this experiment make it doubtful that acid could be the sole cause of pulpal degenerations beneath unlined silicate restorations.

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Effect of Tetracycline on the Transmission of Dental Caries in Rats

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Zipkin, Larson, and Rall[1] demonstrated a reduction in caries activity in white rats whose mothers had received tetracycline (T) during various prenatal and postpartum periods. The teeth of these animals at weaning showed the characteristic fluorescence which has been described by various authors. Keyes[2] found that caries activity might be low to negligible in hamsters whose mothers were fed penicillin before, during, and for 1 week after pregnancy and that several subsequent generations remained inactive without further use of the antibiotic. The present experiment demonstrates that the reduced caries activity as previously described[1] is an antibacterial effect on the oral flora per se and is not associated with the fluorescence in the teeth.

EXPERIMENTAL METHODS

All the animals were of the Sprague-Dawley strain, bred in our laboratory with stock from the N.I.H. colony. In each of the experiments the young were weaned at 21 days of age and housed 2 per cage according to sex. Tetracycline* in distilled H_2O was administered to selected groups of animals as subsequently indicated. All animals were fed caries-test diet 636[3] for 60 days, then sacrificed, the jaws cleaned by dermestid beetles,[4] and the teeth scored for caries according to Keyes.[5]

Experiment A consisted of three groups as follows: (*a*) controls with no exposure to tetracycline; (*b*) rats whose mothers were given 0.25 per cent T in the drinking water from the day of conception until the progeny were 14 days of age; (*c*) one control and one tetracycline-treated animal caged together.

Experiment B consisted of three groups as follows: (*a*) controls; (*b*) mothers and young who were given 0.25 per cent T in the drinking water during the last 7 days prior to weaning; (*c*) one control and one tetracycline-treated animal caged together.

Experiment C consisted of two groups receiving no tetracycline prior to weaning: (*a*) controls receiving distilled water throughout and (*b*) 0.25 per cent T solution during the first 7 days on the caries-test diet and distilled H_2O for the remaining portion of the experimental period.

RESULTS

In Experiments A and B (Tables 1 and 2) the tetracycline-treated animals housed separately were markedly less caries-active than the controls, as indicated by fewer

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carious areas on all tooth surfaces. When the control and the tetracycline-treated animals were housed in the same cage, they all showed caries activity essentially comparable with controls caged separately. Likewise, those rats which received tetracycline solution for 7 days prior to weaning, when housed separately, had fewer lesions than the controls but the same number as the controls when they were housed with the controls. However, in those test animals that were housed with the controls, the lesions did not penetrate as deep into the dentin as in the controls (Table 2).

In Experiment C (Table 3) the animals which consumed tetracycline in the drinking water the first 7 days on the caries-test diet and were separately housed showed significantly less caries activity on the buccolingual and the proximal areas than did the controls. However, the sulcal lesions were essentially the same in both groups.

TABLE 1

EXPERIMENT A: CARIES EVALUATION OF ANIMALS WHOSE MOTHERS WERE GIVEN 0.25 PER CENT TETRACYCLINE (T) IN DRINKING WATER FROM ONSET OF PREGNANCY UNTIL PROGENY WERE 14 DAYS OF AGE

GROUPS	NO. OF RATS	PER CENT OF RATS WITH CARIES	AV. NO. CARIOUS TEETH/RAT	AV. NO. CARIOUS AREAS/RAT					
				Buccolingual		Proximal		Sulcal	
				E*	D_s†	E	D_s	E	D_s
Housed separately:									
Control	34	100	9.8	25.0	15.8	8.4	3.2	4.2	0.1
T-treated	38	84	4.0	6.8	3.0	0.9	0.1	1.9	0.0
Housed together:									
Control	35	100	8.5	19.4	7.9	5.3	0.9	4.4	0.3
T-treated	35	94	9.3	19.4	7.4	7.5	0.8	5.3	0.4

* E = enamel.

† D_s = slight dentinal penetration. Very few lesions penetrated farther into the dentin, and they are not reported separately.

TABLE 2

EXPERIMENT B: CARIES EVALUATION OF ANIMALS GIVEN 0.25 PER CENT TETRACYCLINE (T) IN DRINKING WATER 7 DAYS PRIOR TO WEANING

GROUPS	NO. OF RATS	PER CENT OF RATS WITH CARIES	AV. NO. CARIOUS TEETH/RAT	AV. NO. CARIOUS AREAS/RAT					
				Buccolingual		Proximal		Sulcal	
				E	D_s*	E	D_s	E	D_s
Housed separately:									
Control	19	100	8.7	18.4	11.4	5.3	2.2	5.4	0.0
T-treated	24	83	3.9	4.5	1.7	0.3	0.0	2.9	0.0
Housed together:									
Control	20	100	9.2	21.1	13.5	5.2	1.9	4.9	0.0
T-treated	20	100	8.7	17.7	7.4	4.9	0.7	4.8	0.1

* See footnote to Table 1.

DISCUSSION

The present findings corroborate the caries-inhibiting effect of tetracycline when administered to the rat in the drinking water during gestation and 14 days of lactation, as previously reported from this laboratory.[1] In addition, the present data indicate that tetracycline is effective in reducing caries activity when administered for 7 days immediately prior to or just after weaning. However, rats pretreated with tetracycline and having the typical fluorescence in the tooth, but caged with the control animals, developed caries comparable with the controls. Hence it appears that the caries activity of tetracycline-treated animals remains low as long as they are housed separately, but, if caged with caries-active animals, they become caries-active, presumably as a result of reinfection. These results suggest that tetracycline exerts its effect as a result of its bacteriostatic effect on the oral flora rather than by its incorporation into and modification of the chemical properties of the tooth itself. Although no work

TABLE 3

EXPERIMENT C: CARIES EVALUATION OF ANIMALS GIVEN 0.25 PER CENT TETRACYCLINE (T) IN DRINKING WATER FOR FIRST 7 DAYS ON CARIES-TEST DIET

GROUPS	NO. OF RATS	PER CENT OF RATS WITH CARIES	AV. NO. CARIOUS TEETH/RAT	AV. NO. CARIOUS AREAS/RAT					
				Buccolingual		Proximal		Sulcal	
				E	D_s*	E	D_s	E	D_s
Controls	23	100	11.0	34.9	20.9	11.6	4.6	6.2	0.3
T-treated	22	100	5.2	3.0	0.6	0.3	0.0	5.1	0.1

* See footnote to Table 1.

has been done on the fecal or oral flora of tetracycline-treated rats, these results support the concept of the transmissible nature of caries in rats.[2]

While the animals treated with tetracycline after weaning showed significantly less caries activity on the smooth surfaces, there was no reduction in the sulci. This finding suggests that, once the "cariogenic flora" was established in the sulci, tetracycline administered in the drinking water was ineffective in reducing caries activity in these areas.

Tetracycline was well tolerated in this and a previous study.[1] Since this treatment results in a highly significant reduction in experimental caries activity, it would seem to offer a practical method of depressing the cariogenic flora and thus could be useful in the study of various superimposed factors on experimental caries in rats.

SUMMARY

The addition of 0.25 per cent tetracycline to the drinking water has been associated with a reduction of caries activity in young weanling rats on a caries-test diet when separately housed and administered (1) to the mothers during gestation and the first 14 days of lactation, (2) to mothers and offspring the last 7 days prior to weaning, or (3) to offspring the first week postweaning. Tetracycline-treated rats housed

with untreated control animals during the caries-test period developed caries of the enamel comparable to controls. These findings give further evidence of the transmissible nature of a "cariogenic flora" in the rat and suggest that tetracycline may be used to depress the oral flora in the study of superimposed factors on experimental caries.

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Transitional Ameloblastic Activity Zone in Mice Teeth

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Particular regions of the fibrillar protein enamel matrix in mouse mandibular incisors were compared previously with reference to morphologic differences presumably resulting from administration of dissimilar fluoride compounds and distilled water.[1] Additional studies considered alterations in this matrix formation in mice following injections of bovine parotid gland extracts.[2, 3]

Differences in the biologic activity of ameloblasts in relation to three transitional regions were of particular interest in the above-mentioned investigations.[1–3] These regions were named zones A, B, and C[1] and are briefly explained in this text under "Results." Alterations in the ameloblasts were most striking in the transitional area designated at zone B, for here they became shortened and separated from the underlying enamel protein matrix. This took place after a cessation of secretory activity occurring in zone A.

Events taking place in zone B are believed to be an important step in those calcification processes of enamel that follow soon after matrix formation. It is likewise felt that increases in vascularity and in extracellular fluid in this and adjacent regions give additional evidence to a meaningful metabolic step in enamel development.

Further to evaluate the significance of this area, investigations were instituted for this study in mouse mandibular incisors from birth to 29 days, in order to determine whether this area was present at birth and, if so, what happened concerning it during the first few weeks after birth. It was also of interest to determine whether this area could be found in molar teeth and, if so, what activity and location it assumed as compared with the continuously growing incisors. This paper answers the above questions and considers possible explanations concerning the phenomenon of zone B.

EXPERIMENTAL METHODS

Young adult mice of the Rockland strain were bred, and two to four of their offspring were obtained and sacrificed on a daily basis from birth to 30 days. Such a group representing the entire period constituted one series. These series were repeated at least six times and frequently more. Sex of the animals was also determined, so that jaw sections of males as well as females could be studied histologically.

Mice to be studied were decapitated, and the lower jaws were removed and immediately fixed in Zenker's solution and alcohol or formalin or Carnoy's solution. These different fixatives were used to determine whether the zone in question was related to fixation.

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Sections to be studied were decalcified in formic acid and sodium citrate. Other decalcifying methods were employed (DeCal and nitric acid) but were abandoned because the formic acid and sodium citrate method gave the best results.

Each half of the mandible, after paraffin imbedding, was sectioned serially at 7 μ in a vertical plane through the mid-line, in order to present an entire length of ameloblasts so that their several conversion zones could be observed in relation to the subjacent enamel matrix formation. Staining was usually with hematoxylin and eosin.

Sections were studied mainly with reference to zone B in the incisors. Molar teeth were likewise observed with regard to this particular region. The area in question was considered at birth and after 8, 15, and 29 days. In addition, these same areas in molars are shown at 9 and 15 days after birth.

The following criteria were used to substantiate the presence of this zone: (1) a shortening of the ameloblasts which at the same time maintained a juxtaposition to the stratum intermedium cells (all figures); (2) a separation or space between the basal aspects of the ameloblasts and the matrix (all figures); (3) a morphologic change in the ameloblasts due to a loss or disappearance of the Tomes processes (Figs. 3–7); (4) a directional change in the peripheral border of the fibrillar protein matrix not evident in zone A (Fig. 6); (5) vacuoles or extracellular spaces either between the ameloblasts and stratum intermedium cells or between the ameloblasts or in both locations (all figures); (6) evidence of a greater size or extension of the blood vessels associated with this area (Figs. 1, 3–5); (7) accumulation of acellular residual material in the space between the ameloblasts and the protein matrix (all figures); and (8) compression or depression in the fibrillar protein matrix opposite the concavity formed by the shortened ameloblasts (Figs. 5, 6, and 8).

RESULTS

Three distinct zones of the organic enamel matrix of the mouse mandibular incisor remained after decalcification and are shown in Figure 1 when jaws were sectioned in the manner described above. This specimen was taken from a male mouse 15 days after birth.

This study was concerned primarily with events taking place in the region described as zone B, but for the readers' orientation all three zones are described here briefly.

Zone A—the matrix secretory zone of the ameloblasts—extends from the base of the tooth incisally to its limitation by zone B. Zone B, between zones A and C, is indicated by a shortening of the ameloblasts that creates a space between them and the underlying matrix; this is also called the "zone of compression" by this author. Zone C extends from zone B incisally and is marked by a readjustment in length and a reattachment of the ameloblasts to the protein matrix.

Appearance of zones at birth.—The region designated as zone B is present at birth in the mouse mandibular incisor, usually being small and located about midway in the palisaded ameloblast length but at times being nearer to the incisors' base. Ameloblasts exhibit a concavity here toward the underlying matrix. Figure 2 shows this place in a specimen obtained exactly at birth from a female mouse. Residual material is seen in the area, and it is important to note that no enamel protein matrix is at this time evident, but already there seems to be the formation of a thin layer of material along the dentino-enamel junction. No Tomes processes are seen on the ameloblasts in zone

B, and toward the center these cells are shortest. In addition, adjacent stratum intermedium cells are crowded into fewer layers, whereas in the zone of matrix secretion, zone A, there are more layers in a loose arrangement. Furthermore, in zone B the nuclei of these cells stain darker and have an almost rectangular shape. In zone A these stratum intermedium cells are elongated, and their nuclei are flattened in appearance. It is important to note that between the ameloblasts and the stratum intermedium cells several several vacuolar spaces may be observed. Odontoblasts opposite this region, as compared with other pulpal areas, are not changed. Likewise early dentin

FIG. 1.—All zones 15 days after birth, male mouse. *A* = matrix secretory zone, *B* = matrix compression zone, *C* = matrix reattachment zone, *BV* = blood vessels, *P* = dental pulp and cells, *D* = dentin, *M* = enamel matrix, and *Od* = odontoblasts. (Orig. mag. ×73.0.)

(darker staining) and predentin (lighter staining) formations which are evident in comparison with other pulpal regions do not vary.

Serial sections show that zone B is wider and nearer to the incisal edge at the midline, becoming narrower on each side in a downward slope toward the base of the incisor.

Appearance 8 days after birth.—Zone B, the zone of compression, may become larger and alter its position as the mice grow older. Significantly, the change in position is usually away from the incisal tip rather than toward it. Such changes, however, fail

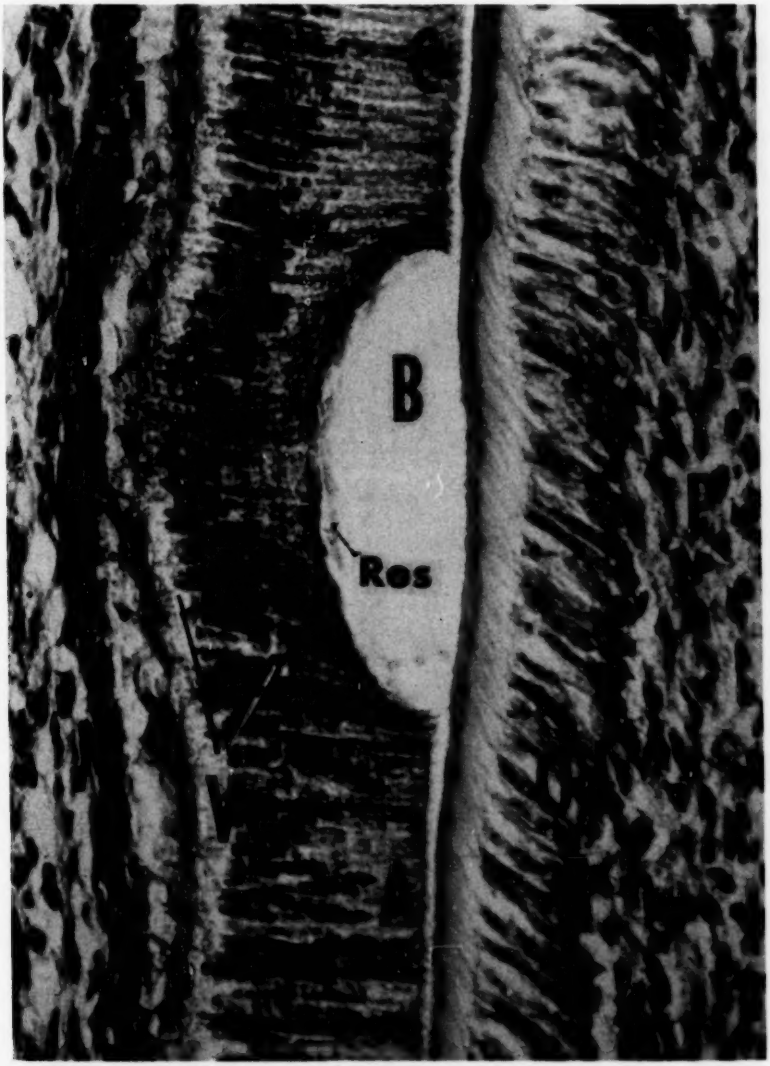

FIG. 2.—Zone B at birth, female mouse. *Vac* = vacuoles and extracellular spaces, *Res* = residual material, *Am* = ameloblasts, and *Si* = stratum intermedium cells. (Orig. mag. ×1,120.)

to correspond consistently with the animals' ages, for zone B may become very wide by 7–8 days and by 16–18 days may sometimes appear greatly reduced in size. This transitional region, designated as zone B, however, persists as a demarcation between the secretory zone A and reattachment zone C.

Zone B is shown at 8 days in Figure 3, in a section obtained from a male mouse. Compression of ameloblasts is quite marked in this instance, and they also appear markedly altered in characteristic morphology. The secretory zone of the ameloblasts, if included, would be at the top of this figure, and at the bottom the zone of reattachment, zone C. Residual material is present on the matrix surface. Opposite this region, pulpal areas remain unchanged or unaffected, but stratum intermedium cells are compressed and present a distinctly different appearance as compared with the other zones.

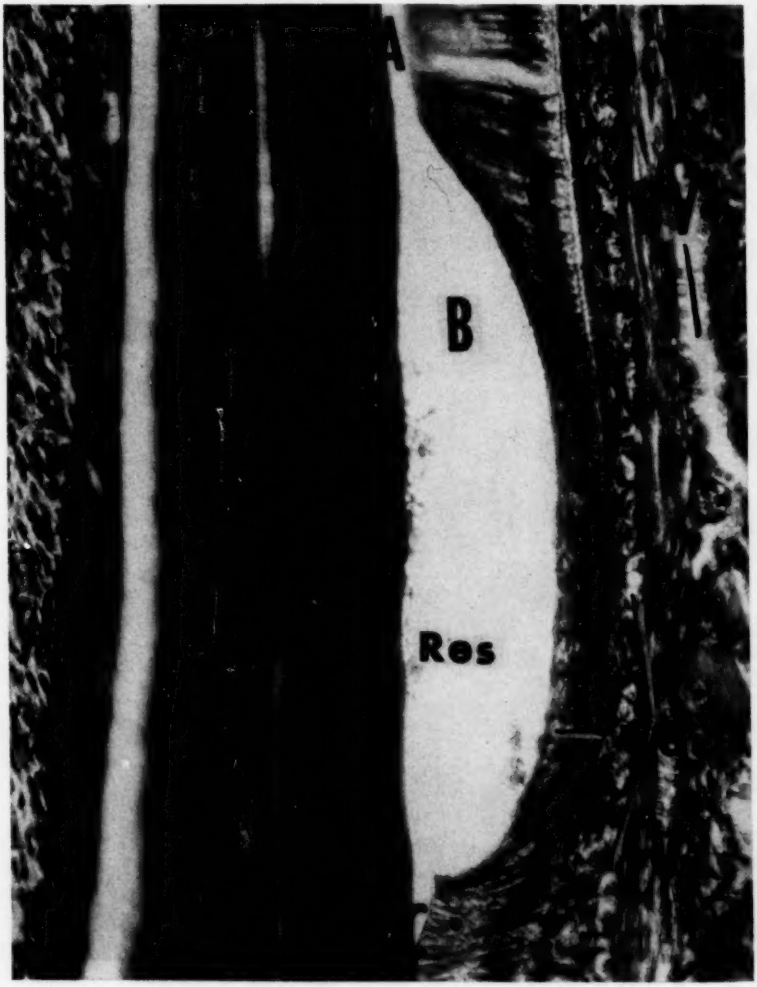

FIG. 3.—Zone B 8 days after birth, male mouse. (Orig. mag. ×450.)

Some small vacuoles appear between the polar aspects of the ameloblasts and stratum intermedium cells. Large blood vessels, not remarkable in appearance, lie beyond the stratum intermedium cells.

Appearance 15 days after birth.—Vascularity, as well as the number and size of vacuolar spaces, usually shows a marked increase from the twelfth to the sixteenth day. Sometimes the picture represented by Figure 4, which is a section from a female mouse, is seen. This shows a double area, the bottom being a regular example of zone B and the upper region depicting a reversed situation with a separation between the stratum intermedium cells and the subjacent ameloblasts instead of a separation from the

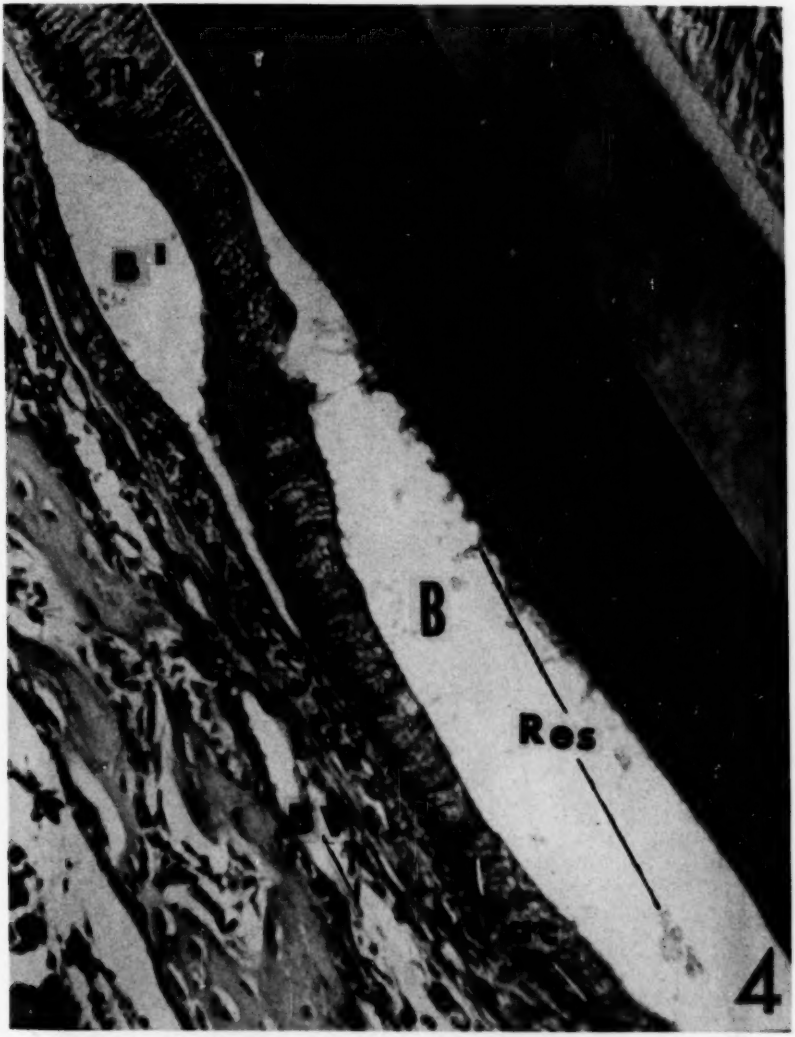

FIG. 4.—Zone B and an auxiliary zone 15 days after birth. B^1 = auxiliary maxtrix compression zone. (Orig. mag. ×450.)

enamel matrix. In both instances, significantly, the ameloblasts are characteristically shortened, which thus rules out that this second region is an artifact. The marked increase in vascular spaces, as well as in vacuolar areas, is likewise striking in this region.

Appearance 29 days after birth.—The forces exerted in zone B may become marked enough to show considerable compression of the protein matrix of enamel, as is demonstrated in Figure 5, which shows a section obtained from a male mouse 29 days after birth. At times there may be more than one of these areas in a single incisor. Zone A is at the top of this figure.

In Figure 6 the lower portion of zone B is seen and a part of the zone of reattachment, zone C. A change in length of the ameloblasts is apparent. In zone C there is a

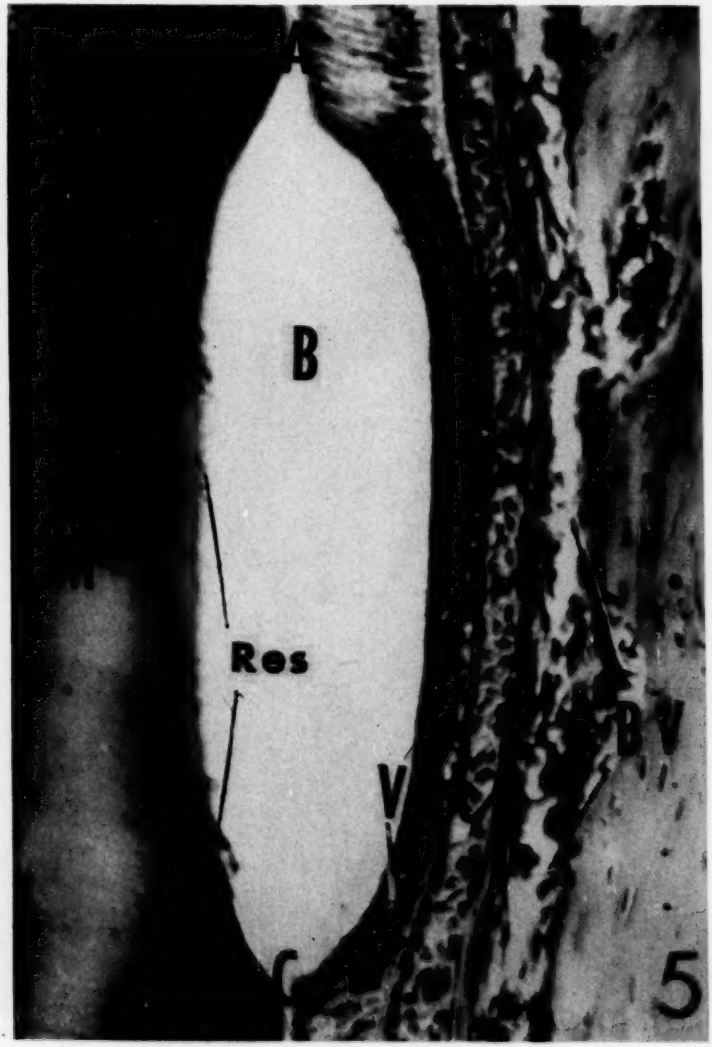

FIG. 5.—Zone B 29 days after birth, male mouse. (Orig. mag. ×450).

lack of complete polarity, which is also obvious in the top part of Figure 2. Residual material and the directional change of the peripheral protein matrix are well depicted.

It is interesting to note that the area described as the "zone of compression," zone B, may also be found in molar teeth of mice during early stages of development. Erupted teeth do not show this area, but it is always noted in definite places along molar cusp surfaces during matrix formation and prior to eruption.

Figure 7 shows this region in two adjacent molar teeth 5 days after birth. Two B zones are located (*top area*) on the distal of the first molar tooth and (*bottom area*) on the mesial of the second molar tooth. Such areas in these teeth at 5 days after birth correspond (at the stage of matrix development) to the area of the mandibular incisor seen in Figure 2. In this instance, however, the mouse was a female. There are some acellular spaces lying near these areas, but neither they nor regional blood vessels indicate any increase in this region at this stage of development.

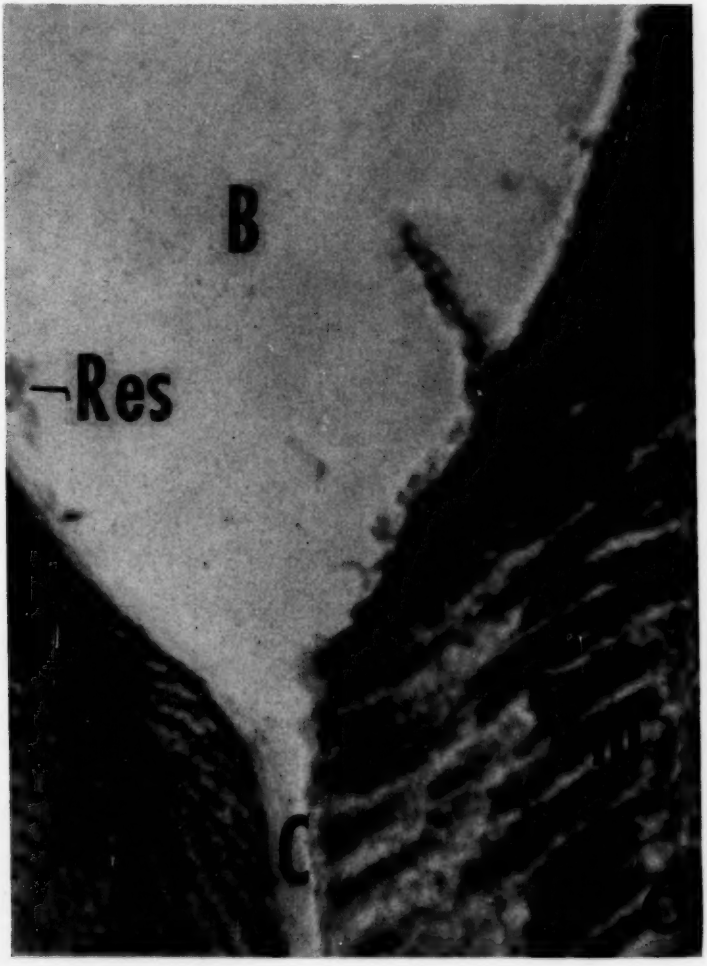

FIG. 6.—Higher power of zone B in Fig. 5 at zone of reattachment. (Orig. mag. ×1,800.)

Figure 8 shows this zone in a third mandibular molar obtained from a male mouse 18 days after birth. Two of the regions called zone B are evident at opposite sides of this tooth, being located in about the same place toward the bottom of the crown. All the characteristics of the "compression zone" as observed in the manidublar incisor teeth are present. Large blood vessels lie near these areas, but increased vacuolar spaces are not marked.

DISCUSSION

One objective of this investigation was to determine whether the transitional region marked by a shortening or concavity of ameloblasts was present at birth in mouse man-

FIG. 7.—First and second molars in mandible, female mouse, 5 days after birth. (Orig. mag. ×450.)

dibular incisors. Attention was likewise drawn to the molar-teeth anlagen, to learn whether here, too, these transitional areas were present. In both instances, these areas were present, persisting in the continuously growing incisors but not in the mature molars.

It has been agreed that initial steps in enamel prism formation follow the secretion of the fibrillar protein matrix,[1, 4–10] and it has also been emphasized that secretory ameloblasts have a definite affinitive relationship with their protein fabrications.[1, 6–8, 11–13] What is not clear, however, is the sequence of events following this initial functional activity.[1, 8, 9, 14, 15]

After the ameloblasts cease their secretory activities in the mandibular incisors of

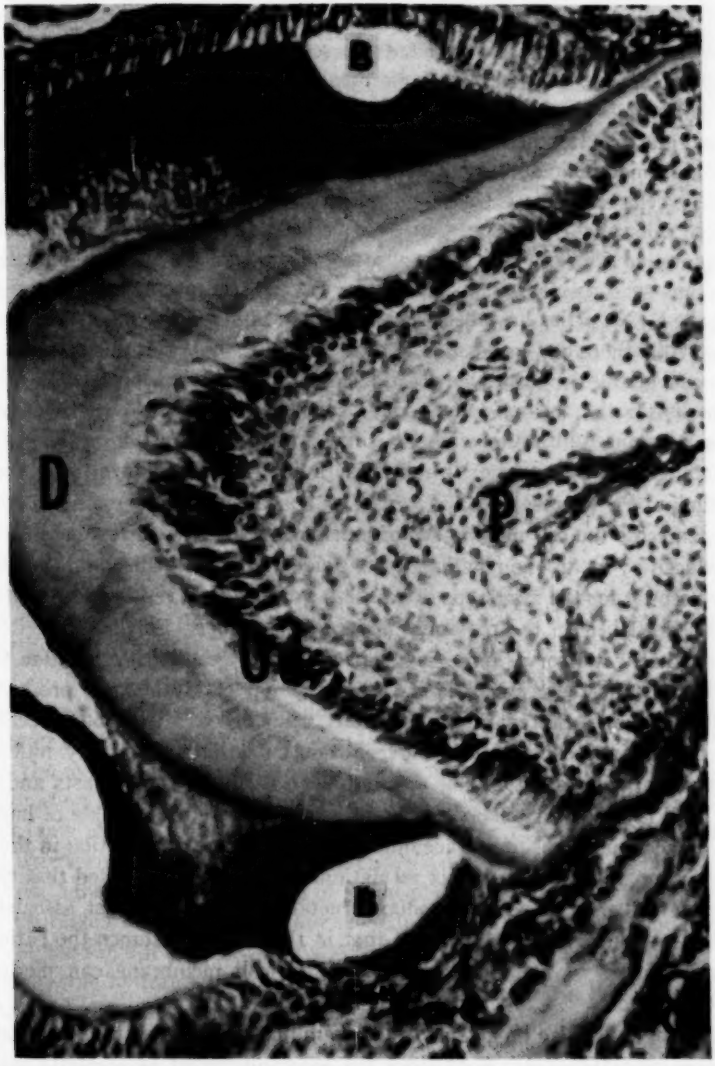

FIG. 8.—Third mandibular molar 18 days following birth, male mouse. (Orig. mag. ×240.)

mice, they occupy the region which has been designated as zone B.[1] Marshland,[7, 8] in his studies of amelogenesis in rat incisors, noted that where the ameloblasts had stopped secreting, there was an artifact which became non-existent upon their reattachment to the protein matrix. He was not convinced, however, of the reality of this region. Levy's[16] interest has recently been aroused by these same areas in mice incisors.

The possibility that this transitional zone in the ameloblasts could have resulted from a stoppage in matrix formation due to metabolic insults or upsets was considered. Furthermore the possibility that this region might have been due to a separation or tearing of tissues was examined. Both these probabilities were ruled out because the several criteria set forth in the "Experimental Methods" section of this paper had to be met. Therefore, several possibilities are presented in an attempt to explain the reason(s) for this region's existence.

The ameloblasts have just completed the secretion of the fibrillar protein matrix. Great energy supplied through metabolic breakdown is required for matrix formation, and this is accomplished by mobilization of bodily resources at particular times for such growth and development.[17, 18] Thus it is logical to assume that, following such a period of high metabolic activity, a depletion or imbalance of certain substances relative to these cells exists. It is therefore reasonable to expect that the K/Na ratio[19, 20] inside these cells might have been changed in accordance with the phenomenon of a Gibbs-Donnan equilibrium[19, 21, 22] because of an unequal distribution of extracellular diffusible ions such as Na^+, Cl^-, HCO_3^-, or H^+. In the presence of these diffusible ions once the K/Na ratios have been attained, intra- and extracellular water movement ceases, and it is, therefore, impossible to draw energy.[21, 22] When this occurs additional energy is unavailable for continued matrix secretion by the ameloblasts. In addition, if phosphate distribution is considered—and it must be with regard to active transport—the Gibbs-Donnan equilibrium might be shifted in either direction,[22] thus accounting for an accumulation of extra- or intracellular fluid. In this instance the accumulation is extracellular.

Water can cross the cell membranes of ameloblasts in either direction as a result of being produced by metabolic reactions on one side and being utilized on the other side. This may account for the numerous extracellular spaces observed between and near the ameloblasts described in the previous[1] and present studies. Furthermore, it must be emphasized that water serves more as a substratum for reactions in solution than as a metabolite and that there are oxidizing enzymes that catalyze water production and hydrolyzing enzymes that catalyze reactions utilizing it.[20] All bodily cells act as osmometers reacting to changes in external concentrations of ions by movement of water but not of solutes across cell membranes.[19, 20, 22] The ameloblasts and/or their associated cell layers of the enamel organ presumably were not capable of handling or transporting accumulations of extracellular fluid at the time of depletion in the energy-providing system which aided matrix formation. It has been postulated that the active carrier across cell membranes is a reduced metal-containing catalyst associated with the energy-providing system.[24] It is felt that in the present instance the cation is not bound, and because of metabolic alterations, the cell membrane can no longer be crossed at this time.

Consideration must also be given to the possibility that the matrix could handle the accumulation of extracellular fluid since the energy-depleted ameloblasts are at this

time unable to do so. In the extracellular fluid alterations that will take place, the newly formed protein matrix could possibly exert an attracting force. This attraction might result from certain regions of highly organized (non-random) molecular structures which, at specific points in these fibrous proteins, have particular anions loosely bound to polypeptide chains.[25, 26] The protein-bound -S- molecules might play a part, since it is known that the keratin subgroups of the protein enamel matrix are rich in sulfur.[10, 28] The attraction, however, must be from some molecule other than the sulfur molecule, which in this instance is presumed to be saturated and firmly bound. This affinity, however, could be considered as the result of total forces exerted between two small neighboring regions (gas or fluid) and dependent upon the number of molecules in the region.[25, 29] In this case the greater number of molecules must be within the matrix. The concentration of extracellular fluids could thus be attracted by this specific thing. Furthermore, it is likewise known that water molecules have considerable dipole moments, with the charged parts inducing an opposite charge in a neighboring molecule, resulting in an attraction between the two.[19, 22] While at one time water or fluid could be atracted to the matrix, it is also necessary to consider an opposite reaction taking place.

This newly formed protein matrix should be rich in water presumably with K^+ and Na^+ as well as Ca^{++} and HCO_3^- and $HPO_4^=$ ions in solution. By loss of water from the matrix, a subsequent increased aggregation of ions should result within the matrix compartments and could thus cause a transfer from a liquid to a solid phase. Consequently, the organic matrix may at this point institute the formation of crystals along its fibrillar structure, conceivably following the removal from it of specific amounts of water. All this is in keeping with a "second concept" of mineralization and, as Glimcher also states, in order for such a mechanism to operate, solutions that will form crystals must be in metastable equilibrium.[30] Either by the attraction of extracellular fluids to this area or by removal of water from the fibrillar protein matrix or gel, solute adjustments must take place if crystals, such as enamel in this instance, are to begin formation along the fibrillar compartments of the protein matrix. All this is in keeping with present concepts of crystal induction.[31] Thus initiation of crystallization, which will soon be followed by recrystallization, should begin to take place within the matrix, and immediately prior to this there must be an adjustment of metastable solutes as the associated processes of mineralization progress in zone B.

There may, in association with zone B, be a combination of the above factors either acting concurrently or in sequence. Thus the above reasoning follows suggestions that the apatite crystals of enamel are closely associated with a structural and crystalline protein as the mechanism of crystal induction take place.[14, 30]

In explanation of this ameloblastic transitional zone, it appears likely that its presence marks one of the stepwise mineralization processes taking place at this stage of enamel matrix formation. According to the above reasoning, the energy-depleted ameloblasts cannot handle the accumulation of extracellular fluid that is either drawn to this region or expressed from the newly formed matrix at this time in solution adjustments that will set the stage for calcification and crystallization of the enamel protein matrix. That considerable hydraulic pressure is exerted at such times is revealed by the compression of both the matrix and the ameloblasts.

Further studies must involve microanalysis of this region by microtechniques, as well as sectioning and study by electron microscopy.

SUMMARY

The ameloblastic transitional region known as zone B or the "zone of compression" is present at birth in the mandibular incisors of mice. This same region is found in anlagen of mandibular cheek or molar teeth of these animals. In each developing molar tooth one of these zones is associated with each cusp, and cross-sections of these teeth show them in similar positions on opposite sides of the crown. While this region persists in continuously growing mandibular incisors, it disappears in the cheek or molar teeth as they reach maturity. Usually only one of these regions is present in the incisors, but at times there may be an auxilliary region that fits all the criteria for this zone. This zone may vary in its size and position in relation to the length of the palisaded ameloblasts of the incisors, but it is always present. Considerable force appears to be exerted against the matrix in this region. This pressure is manifested by a compression of the matrix. This force is presumed to be of a hydraulic nature and related to the mineralization and crystallization processes evidently scheduled to take place at this time in the enamel matrix. It is felt that this region and adjacent regional extracellular spaces represent attempts at solute adjustments that will initiate crystal formation along the fibrils of the newly formed enamel protein matrix.

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Ionized Calcium in Saliva

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The concentration of ionized calcium in the saliva is of interest in respect to (1) the initiation and propagation of the carious process and (2) the deposition of dental calculus. In the following, measurements of the ionized calcium concentration in saliva are reported. In general, the method consists in the addition of a dye—murexide—to the saliva and the measurement of the optical absorption, the principle of the method depending on the fact that murexide combines with calcium according to the mass law and the fact that murexide combined with calcium has a different optical absorption from murexide not so combined. The method was described by Raaflaub[1] and has been applied to the ultrafiltrates of serum by several investigators.

Using brackets to denote molar concentrations, T.M. is total murexide, M is uncombined murexide, CaM is murexide complexed by calcium, [CaM] is α [T.M.], Ca is free calcium ions, T.C. is total calcium, Em is the molar absorption coefficient of uncombined murexide, Eca that of the calcium murexide complex, Et that of murexide at the isobestic point. The method is as follows.

In preliminary experiments on solutions having a specific concentration of murexide, [T.M.], and a range of ionic strengths and pH values corresponding to those of saliva, the following are measured: (1) the absorption at 490 millimicrons and at 530 millimicrons in the presence of a relatively high concentration of calcium, Aca = [T.M.] Eca; (2) the absorption at the same wave lengths in the absence of calcium, Am = [T.M.] Em; (3) the absorption at the same two wave lengths of calcium solutions of such concentration that [CaM] is of the same order of magnitude as [M], the absorption being Ax = [M] Em + [CaM] Eca; (4) the wave length of the isobestic point and the absorption at the isobestic point, At = [T.M.] Et. (As a result of these measurements Eca, Em, and Et are determined.)

It can be shown that

$$\frac{Ax - Am}{Aca - Am} = \alpha, \quad \begin{array}{l}\text{where all the absorptions are those} \\ \text{at either 490 or 530 millimicrons,}\end{array} \tag{1}$$

and that, if K is the dissociation constant of the reaction $Ca + M \rightleftharpoons CaM$,

$$K = [Ca]\frac{1-\alpha}{\alpha}. \tag{2}$$

In the preliminary experiments, the determination of α allows [Ca] to be calculated from [T.C.], which is known, and thus K is determined over a range of pH values and ionic strengths.

There are determined on a saliva sample (1) the absorption at 490 and 530 millimicrons, Ax; (2) the pH and ionic strength, μ (the latter estimated), and from these,

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by use of the data on pure solutions, the isobestic point; (3) the absorption at the isobestic point and, from this, using Et, [T.M.], and from this, by using Eca and Em, Aca and Am. From these data α is calculated by equation (1) and from α and the value of K at the pH and ionic strength of the sample in question, the value of [Ca], the ionized calcium concentration.

There result two values of the ionized calcium concentration because α is independently determined by the values of Ax, Aca, and Am at 490 and at 530 millimicrons, respectively. The two wave lengths are so chosen that the difference between Eca and Em is maximal and the two determinations serve as good checks on each other because at 490 millimicrons Eca is greater than Em but at 530 millimicrons it is less than Em.

The ionic strength of the saliva is known only approximately. From conductivity and total base measurements on fifteen salivas by Schmidt-Nielsen[2] and with employment of a factor of 1.12 calculated by Ericsson[3] to account for the divalent ions present, the ionic strength of the saliva can be put at about 0.03, with an average deviation from this value of about 10 per cent. The largest deviations in Schmidt-Nielsen's series was 30 per cent.

Though the amount of murexide added to the saliva is weighed, more consistent results have been obtained by estimating [T.M.] (and from this Am and Aca) by measurement of the absorption, At, at the isobestic point. The deviation between estimates of [T.M.] by weight and by absorption are often 1 per cent and occasionally as high as 3 per cent. The cause of the discrepancy is not certain.

EXPERIMENTAL METHODS

Reagents.—Murexide: ammonium murexide was made from alloxantin by the method of Davidson.[4] It was stored in the dark *in vacuo*. 24.0 mg. of the solid and 5.0 ml. of "tris" buffer, pH 7.37, were made up to 200 ml.

Buffer: 1 M tris (hydroxymethyl) aminomethane was adjusted to the desired pH with HCl. The ionic strength of the solutions was altered by altering the strength of the buffer.

Calcium standards: A 0.4 molar calcium chloride solution was made by dissolving 10.00 gm. calcium carbonate in 17.0 ml. of concentrated HCl and adding water to 250 ml. Lower dilutions were made from this.

Ethylenediaminetetraacetate: 0.336 gm. of disodium dihydrogen ethylenediaminetetraacetate dihydrate and 0.6 ml. of normal sodium hydroxide were diluted to 100 ml.

All these reagents and dilutions therefrom were made with distilled water which had been deionized in a mixed-bed deionizer. All glassware was soaked in dichromate–sulfuric acid cleaning solution, then rinsed with tap water, distilled water, 2 per cent HCl, distilled water, and, finally, deionized water.

Method.—The values in Table 1 were determined on pure solutions by the method described above with solutions having the following values for [T.C.]: 0, 4×10^{-2}, 4×10^{-3}, 1×10^{-3}. The values in the table are averages from at least four determinations.

Saliva was collected under a layer of toluene; the pH was measured on an aliquot, and the remainder was centrifuged, still under toluene and from the supernatant aliquots taken for ultrafilterable calcium if this was measured. The supernatant remaining was filtered through one or sometimes two filter papers (SS No. 595), and

samples of the filtrate taken for total calcium analysis. The pH of the remaining filtrate was adjusted to the original value by bubbling 6 per cent CO_2 in nitrogen through it. To 5.5 ml. of the resulting fluid was added 0.500 ml. of murexide solution. (Final concentration of dye, 4.22×10^{-5} M.) The two fluids were mixed in a 13 × 100-mm. tube and then read in a Zeiss spectrophotometer in cuvettes of 10-mm. light path which were covered with waxed paper. The absorption was read at 490, 508, 510, and 530 millimicrons against the saliva without murexide as a blank. The calculation of ionized calcium was made as outlined above. Total calcium was determined by the method, with ashing, of Harnach[5] and ultrafilterable calcium by filtration through cellophane.

RESULTS

The results of twenty-one measurements of ionized calcium in saliva are given in Table 2. The upper figure for the ionized calcium for each sample is that calculated

TABLE 1

MOLAR ABSORPTIONS (ACA, AM, AT) WERE OBTAINED AS DESCRIBED IN TEXT, TOTAL MUREXIDE CONCENTRATION BEING 4.22×10^{-5}

pH	μ	$K \times 10^3$	Aca 490	Aca 530	Am 490	Am 530	Isobestic Point (mμ)	At
6.6	0.018	2.66	0.808	0.279	0.441	0.601	508	0.581
6.6	0.030	2.93	0.799	0.279	0.435	0.595	508	0.576
6.6	0.040	3.25	0.780	0.274	0.430	0.580	508	0.559
6.88	0.018	2.54	0.803	0.284	0.444	0.602	508	0.578
6.88	0.030	2.63	0.791	0.280	0.438	0.594	508	0.573
6.88	0.040	3.05	0.772	0.271	0.427	0.578	508	0.560
7.20	0.018	2.18	0.812	0.294	0.436	0.601	510	0.581
7.20	0.030	2.41	0.791	0.285	0.432	0.589	508	0.571
7.20	0.040	2.63	0.799	0.289	0.432	0.591	508	0.569
7.40	0.018	1.91	0.802	0.294	0.436	0.599	510	0.578
	0.030	2.17	0.785	0.285	0.430	0.582	508	0.574
	0.040	2.31	0.791	0.290	0.434	0.586	510	0.568
7.80	0.018	1.25	0.804	0.311	0.431	0.592	510	0.577
7.80	0.030	1.44	0.781	0.295	0.429	0.579	510	0.575
7.80	0.040	1.35	0.791	0.306	0.430	0.587	510	0.568

from values of Am, Aca, and Ax at 490 millimicrons, and the lower figure is calculated from values for the same quantities measured at 530 millimicrons. The average deviation between two members of such a pair is 5.9 per cent, and this is probably a fair indication of the accuracy of the method. The ionized calcium amounts to from 73 to 102 per cent of the ultrafilterable calcium. Apparently, saliva contains, in some cases, a calcium-complexing substance of low molecular weight. The ionized calcium is 48–70 per cent (average 60 per cent) of the total calcium in the eleven samples in which both were measured.

DISCUSSION

The results indicate that the ionized calcium of the saliva is roughly about half the total calcium. Our knowledge of the solubility of hydroxylapatite is not, it seems at present, in such a state that it can be applied in the form of precise figures to the problem of dental caries. The saliva is probably very nearly saturated, under normal

TABLE 2

CONCENTRATION OF CALCIUM IN MG. PER 100 ML.

Subject	Specimen	pH	Total	Ionized	Ultra-filterable
K.P.	.....	7.14	5.64	3.85 3.99	4.08 .....
M.L.	1	7.35	5.32	3.21 3.78	4.04 .....
	2	7.33	.....	3.31 3.23	....
A.H.	1	7.70	5.40	2.74 3.05	3.92 .....
	2	7.72	.....	2.89 2.94	....
R.T.	1	7.56	4.68	2.98 3.14	3.00 .....
	2	7.62	.....	2.83 2.96	....
M.	1	7.20	5.00	3.37 3.50	4.48 .....
	2	7.72	5.72	2.65 2.85	4.16 .....
T.C.	1	7.70	5.48	2.69 2.69	3.28 .....
	2	7.72	.....	2.45 2.58	....
F.H.	1	7.60	4.64	2.82 2.78	....
	2	7.62	.....	2.78 2.74	....
L.L.	1	7.10	5.40	3.12 3.37	....
	2	7.08	.....	3.09 3.34	....
F.H.	1	7.70	5.60	2.80 2.74	....
	2	7.70	.....	2.76 2.83	....
L.L.	1	6.94	5.32	3.19 3.79	....
	2	6.94	.....	3.30 3.74	....
S.S.	1	7.72	5.72	2.65 2.85	4.16 .....
	2	7.69	.....	2.68 2.73	....

conditions, in respect to the enamel, as is shown by the fact that the teeth do not dissolve. Our most precise figures for the ionic concentrations involved in the production of caries are probably those obtained from the production of caries in vitro.[7]

SUMMARY

In 21 samples of saliva from 9 subjects, analyses of ionized calcium appeared to give consistent values. The ionized calcium was about 60 per cent of the total calcium and 70–100 per cent of the ultrafilterable calcium.

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The Effect of Longitudinal Restrictive Force on Hygroscopic Expansion

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Shortly after his discovery of the phenomenon of hygroscopic expansion, Scheu[1] found that the exertion of force on the setting investment reduced the hygroscopic expansion. Skinner[2] had found that force reduced normal setting expansion. The effect of restrictive forces on hygroscopic expansion has been considered by many investigators and emphasized especially by Docking[3] and by Skinner and Degni.[4]

We attempted to measure the effect of horizontal longitudinal force on hygroscopic expansion and setting expansion of one gypsum investment in a direction against this force. Measurements were also made on the vertical expansion in a direction perpendicular to the force. The effect of longitudinal force on the rate of expansion in the early stages was also measured. An attempt to determine the hygroscopic expansion under vacuum was not successful.

MATERIALS AND METHODS

The investment used contained hydrocal 35 per cent, cristobalite 10 per cent, silica 55 per cent, and chemical additives none. The investment was used at a $W/P = 32/100$; it was spatulated mechanically 30 seconds at 650 r.p.m.; the hygroscopic water was added 5 minutes after mix; and the Vicat setting time was 11 minutes.

The apparatus used for testing hygroscopic and setting expansion is simple to operate and has been used in the control laboratory for many years, having been originally designed by Mr. T. E. Moore. It consisted of a glass plate with one fixed metal stop for confining the specimen at one end and a moveable metal slide contacting the dial gauge which confined the investment at the other end. The sides were confined by glass plates. The specimen was 100 mm. long and 1 square inch in cross-section. The dial gauge was calibrated in millimeters, so that the dial read directly in percentage. For regular setting expansion tests, the area was lined with tin foil or a rubber dam material; and for hygroscopic expansion, it was lined on the bottom and two sides with wet asbestos sheet. Water may be added to the surface from burettes (Fig. 2). The unweighted equipment was used for a control test on each mix of investment.

Force was applied on the setting investment by placing a cord around the movable stop and passing it over the rests and attaching a container to the looped cord. Various weights were placed in the container, producing longitudinal force on the setting investment. There was no confinement in the vertical direction; and in the transverse direction, the confinement was limited to the small glass slabs.

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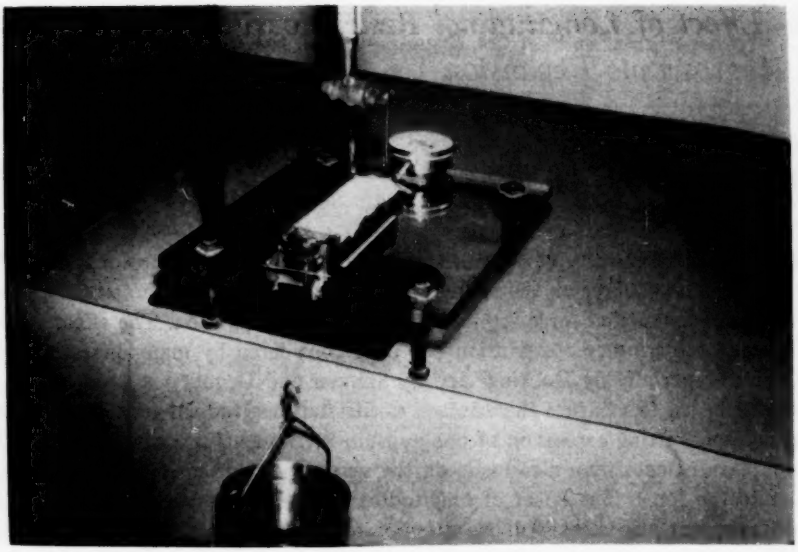

FIG. 1.—Equipment for measuring the setting expansion and the hygroscopic expansion while the investment is under horizontal force. Force is applied by adding weight to the container.

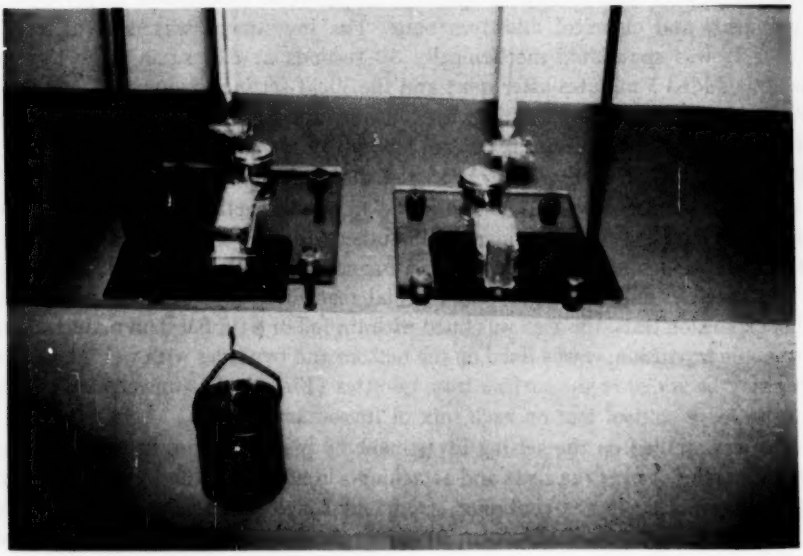

FIG. 2.—Equipment for measuring the expansion, both with and without force exerted on the investment. This provides a control test for each mix.

RESULTS

Force necessary to move the sliding stop and operate the dial gauge with the spring removed was found to be sufficiently restrictive to prevent some hygroscopic expansion, and a counterbalance weight was attached (Fig. 3). The force of friction of the investment against the wet asbestos was not considered until the data were analyzed.

During the tests, a wire was placed in a vertical position at the center of the specimen and a 40-power microscope with a filar micrometer eyepiece was focused on the

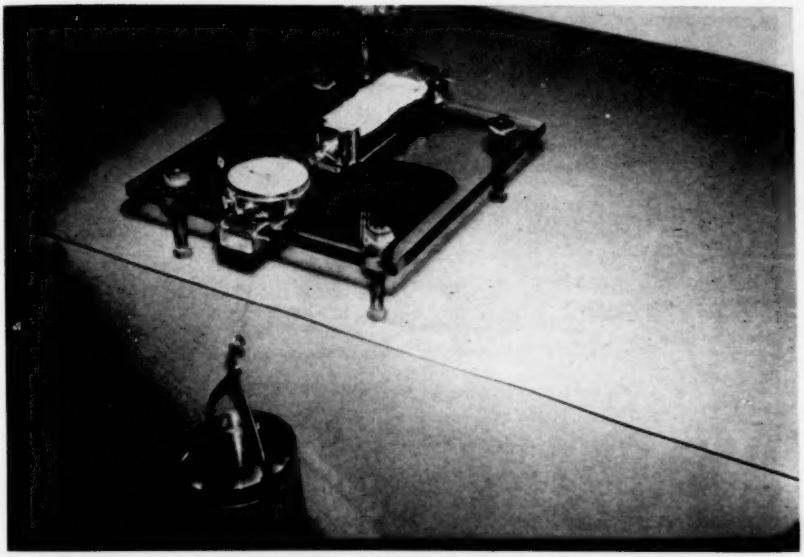

FIG. 3.—Equipment for measuring expansion with the force exerted by moving weight and the dial-gauge counter balanced by weight in the container.

TABLE 1

EFFECT OF LONGITUDINAL FORCE ON SETTING EXPANSIONS OF INVESTMENT*

WEIGHT (GM/CM²)	HYGROSCOPIC EXPANSION		SETTING EXPANSION	
	Rectangular Trough	Wire	Rectangular Trough	Wire
0	2.45	3.5	0.65	0.71
25	1.93	3.3	0.63	.....
50	1.56	.....	0.63	.....
100	1.39	3.3	0.56	.....
200	1.22	3.3	0.52	.....
400	1.04	3.3	0.51	.....
800	0.78	2.9	0.43	.....
1,000	0.76	2.9	0.41	0.63

* Standard deviation (sigma) = 0.13 hygroscopic expansion; $\sigma = 0.04$ setting expansion. Setting time: Vicat, 11 minutes.

end of the wire (Fig. 4). Thus it was possible to measure the vertical expansion coincidentally with the horizontal expansion. In all cases the percentage expansion obtained on the wire tests was much greater than that recorded on the dial gauge.

Table 1 shows the data obtained by using forces from 0 to 1,000 gm/cm^2 on the dial-gauge and the wire method (shown graphically in Fig. 5). On both the dial-gauge test and the vertical wire test, the hygroscopic expansion was obtained by keeping water on the surface of the investment during the entire 2-hour period. Regular setting expansion was obtained with a rubber dam liner and no water added.

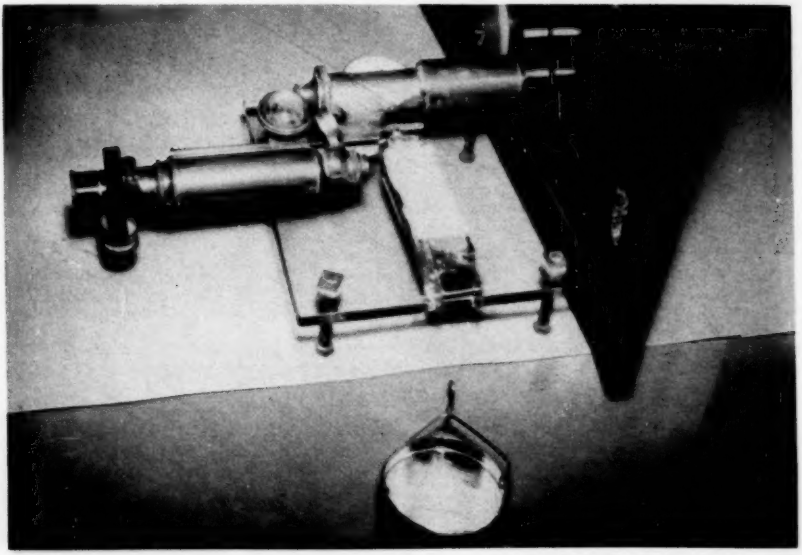

FIG. 4.—Equipment with a micrometer microscope attachment. Longitudinal expansion may be measured on the dial gauge, and vertical expansion by using the wire method on the microscope. Both expansions are measured concurrently.

DISCUSSION

As the weight approached 1,000 gm/cm^2, the dial micrometer reading for hygroscopic expansion approached the value for regular setting expansion with zero force. Inasmuch as 1,000 gm/cm^2 is approximately 1 atmosphere, it seemed possible that the correlation might be significant. This led to an attempt to determine the hygroscopic expansion under vacuum. Unfortunately, the vacuum removed air and possibly water vapor from the investment, causing the material to froth and producing a meaningless reading. Even when the mixed investment was subjected to high vacuum before placing in the apparatus, reproducible results were not obtainable. Every dial-gauge reading obtained showed greater hygroscopic expansion under vacuum than at normal atmospheric pressures.

Unexpected results were obtained when the rate of hygroscopic expansion was determined in the initial stages. The rate of expansion in percentage per minute is shown in tabular form (Table 2) and graphically (Fig. 6). As will be noted, the graph in the initial stages of expansion is almost linear, and the slope is considerably greater when

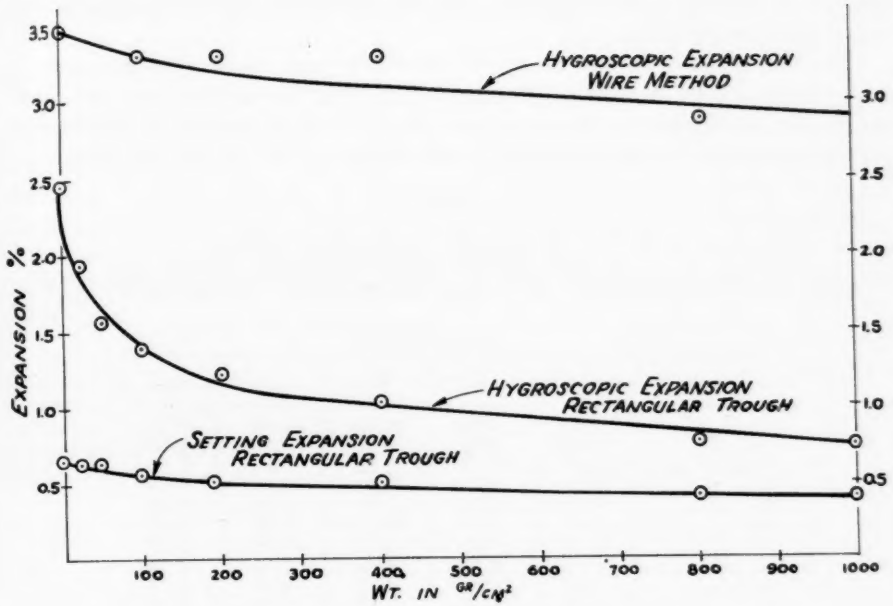

FIG. 5.—Effect of force on expansion of investment

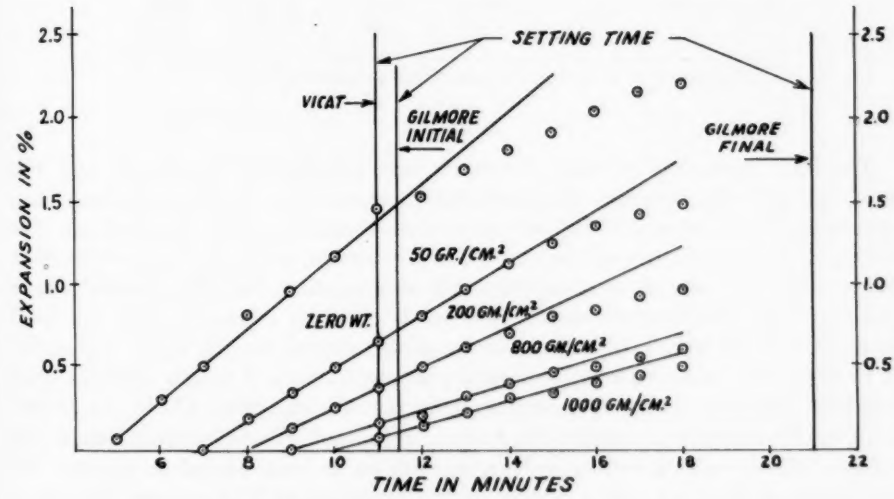

FIG. 6.—Effect of force on rate of hygroscopic expansion

less force is exerted on the investment. Also, the investment started to expand sooner, which might be expected.

From the change in the rate of the setting reaction when force is applied on the investment, some conclusions might be possible regarding the type of physical or chemical reaction responsible for the hygroscopic effect. It may be possible to determine the type of reaction which causes the hygroscopic expansion. This has not been done.

TABLE 2

RATE OF HYGROSCOPIC SETTING EXPANSION BY RECTANGULAR TROUGH METHOD*

MINUTES	WEIGHT (GM/CM²)				
	Zero	50	200	800	1000
5	0.07	0.00	0.00	0.00	0.00
6	0.30	0.00	0.00	0.00	0.00
7	0.50	0.01	0.00	0.00	0.00
8	0.81	0.19	0.00	0.00	0.00
9	0.95	0.34	0.13	0.00	0.00
10	1.17	0.49	0.25	0.07	0.00
11	1.45	0.65	0.37	0.16	0.08
12	1.52	0.81	0.50	0.20	0.16
13	1.68	0.91	0.61	0.32	0.23
14	1.80	1.11	0.69	0.40	0.31
15	1.97	1.24	0.80	0.46	0.34
16	2.04	1.35	0.84	0.50	0.40
17	2.15	1.42	0.92	0.55	0.44
18	2.20	1.49	0.96	0.60	0.50
Slope (%/min).	0.22	0.16	0.12	0.08	0.07
2 hr	2.48	1.60	1.30	0.80	0.77

* Setting time: Vicat, 11 minutes; Gilmore, initial 11.5, final 21.0.

SUMMARY

The force apparently prevents the water from entering the investment, and it is highly possible that the excess of hygroscopic expansion over normal setting expansion is completely independent of the setting reaction of calcium sulfate hemihydrate. The setting reaction might be considered a chemical reaction, while the hygroscopic effect may be the result of a physical reaction, such as absorption. Possibly, chemically pure calcium sulfate hemihydrate would show no hygroscopic expansion, and the small amounts obtained from hydrocal, densite, or plaster of paris may be due to impurities. It is difficult to obtain pure calcium sulfate hemihydrate, as it usually contains some anhydrite and some dihydrate as well as minor foreign impurities. (*Note:* An investment for chrome castings exhibits hygroscopic expansion. This investment contains no calcium sulfate, and the setting reaction depends on an oxide-phosphate reaction. An investment of this type with a normal setting expansion of 0.15 per cent may show hygroscopic expansion of 0.85 per cent.)

Longitudinal force exerted on one investment during setting indicated a marked decrease in the longitudinal hygroscopic expansion, especially in the lower ranges below 100 gm/cm², but it takes more than 1 atmosphere to reduce the hygroscopic expansion

approximately to the value of the setting expansion. Apparently, hygroscopic expansion has adequate strength to move invested wax patterns. Force has a much smaller effect on the normal setting expansion.

Using the wire method,[5] a definite reduction in hygroscopic setting expansion was exhibited in a vertical direction, although not nearly so pronounced as in the horizontal direction, using a balanced dial gauge. The wire method always indicated a greater percentage expansion than that of the balanced dial gauge, possibly because of the restrictive effect of the friction of the investment on the asbestos liners. By extrapolation of the curve for hygroscopic expansion (rectangular trough) to the termination of the curve hygroscopic expansion (wire method), this force might be as little as 10 gm/cm^2 or less.

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The Effect of Certain Additive Agents on the Physical Properties of Zinc Oxide–Eugenol Mixtures

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The palliative effect of zinc oxide and eugenol on the pulp, when used as a base or temporary filling, has long been recognized.[1–5] From this standpoint it is markedly superior to zinc phosphate cement. Likewise the initial adaptation to the cavity walls may be better,[6, 7] as well as the solubility in dilute organic acids.[8] However, longer setting time and inadequate strength have prevented their wider use as a permanent cementing material.

Various accelerators have been employed to decrease the setting time. Chlorides, resin, acetates, and nitrates have all proved successful in accelerating the set;[9–12] however, little research has been conducted to determine the exact mechanism of their behavior or the influence of varying concentrations and mixtures of these agents on the other pertinent physical properties.

The effect of additives employed to increase the strength or hardness of zinc oxide–eugenol mixtures is even more obscure. Use of zinc oxide which has been made by the thermal decomposition of zinc carbonate at 350° C. appeared to result in a more rapid set and somewhat harder and stronger mass.[10, 12] It has been reported[13] that addition of up to 10 per cent polystyrene to the eugenol increases adhesion and "edge strength," although these properties were measured only by digital manipulation of the set cement. Addition of dicalcium phosphate has been suggested as another method for doubling the compressive strength.[14]

Following the chelation theory[15] for the setting reaction between zinc oxide and eugenol, a survey of approximately 50 agents which might produce a chelate reaction with zinc oxide showed that *o*-ethoxybenzoic acid (EBA) was the most promising.[16] A liquid containing 75 per cent EBA and 25 per cent eugenol mixed with a powder of 68.8 per cent zinc oxide, 30.6 per cent silica, and 0.6 per cent zinc acetate produced strengths as high as 10,300 pounds per square inch. However, the solubility in distilled water was over 3.0 per cent. The amount of EBA used appeared to be directly related to the increase in solubility.

Thus a review of the literature reveals that certain manufacturing processes for zinc oxide powder and the use of various additive agents may improve certain physical properties of zinc oxide–eugenol cements, such as setting time and strength, yet they may simultaneously have a deleterious effect on other desirable properties, such as solubility. Furthermore, the additives investigated have generally been studied individ-

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ually, at relatively few concentrations, and not in combination with each other. Since certain of these agents have showed particular promise, the purpose of this investigation was to determine the influence of selected additives, in varying percentages and combinations, upon the physical properties of zinc oxide–eugenol.

EXPERIMENTAL METHODS

Physical tests.—Compressive strength and solubility were the two principal physical properties studied, while film thickness and setting time were measured with the most promising combinations. Compressive strength was conducted in accordance with A.D.A. Specification No. 8 for zinc phosphate cement.[17] A minimum of five specimens was tested for each experimental composition.

For comparison with published data on mixes of pure zinc oxide–eugenol and zinc phosphate cement,[8, 18] solubility in distilled water was determined by a modification of the specification test. The essential difference in the test method is in specimen size and use of fresh distilled water each day during the 7-day immersion period. The results shown are the mean for three specimens.

Standard mix consistency and setting time were measured by the conventional specification tests. Setting times of mixtures that require over 10 minutes to harden are difficult to determine. The mass may harden within a few minutes, but even after hours the end of the needle still makes a perceptible circle. In those cases, the setting time is merely referred to as 10+ minutes.

Because of the rapid set of several of the mixtures used, the film thickness could not be determined by the specification test. Thus two alternative methods were employed. In the "indirect" method, a standard consistency mix of zinc phosphate cement was placed between two 2 × 2 glass plates, and the 33-pound load was applied 2 minutes after initial spatulation. The load was removed at the end of 8 minutes, and the diameter of the specimen was measured. An aliquot of the zinc oxide–eugenol mixture equal to the zinc phosphate cement was placed between the plates and tested in a comparable manner. By comparison of the diameters of the specimens, the relative film thickness could be determined.

In the "direct" method, the specification test was modified by subjecting the cement to the 33-pound load at the end of 2 rather than 3 minutes after initial spatulation. An average of three such tests is reported to the nearest 5μ. The data reported were secured by this method, while the "indirect" measurements served primarily as a check and to indicate the general handling characteristics.

Irritational reaction test.—Biological evaluation of dental materials is becoming increasingly important. There is evidence that additive agents added to dental cements and pulp-capping materials may considerably alter the pulpal effects of the basic material itself.[1] Thus certain of the experimental formulations were studied for inflammatory reaction by subcutaneous placement of specimens in the backs and abdomens of rats. The rats were sacrificed at 2, 16, and 32 days, and the connective tissue reaction around the implants was studied microscopically. This type of test can be well controlled and has been shown to be an excellent method for grading the relative effects of dental materials on tissue.

Materials.—Twenty-six mixtures of zinc oxide and eugenol with various agents added in varying combinations and concentrations were tested. Naturally, a vast

TABLE 1

FORMULAS EMPLOYED IN THIS INVESTIGATION: MATERIALS

A.....	Powder: ZnO; liquid: eugenol
B.....	Powder: ZnO* prepared from zinc carbonate; liquid: eugenol
C.....	Powder: ZnO; liquid: 98% eugenol, 2% polystyrene
D.....	Powder: ZnO; liquid: 95% eugenol, 5% polystyrene
E.....	Powder: ZnO; liquid: 90% eugenol, 10% polystyrene
F.....	Powder: 67% ZnO, 30% fused silica, 3% zinc acetate; liquid: eugenol
G.....	Powder: 67% ZnO, 30% fused silica, 3% zinc acetate; liquid: 98% eugenol, 2% polystyrene
H.....	Powder: 67% ZnO, 30% fused silica, 3% zinc acetate; liquid: 95% eugenol, 5% polystyrene
I.....	Powder: 67% ZnO, 30% fused silica, 3% zinc acetate; liquid: 90% eugenol, 10% polystyrene
J.....	Powder: 70% ZnO, 25% fused silica, 5% polystyrene; liquid: 60% EBA, 40% eugenol
K.....	Powder: 70% ZnO, 25% fused silica, 5% polystyrene; liquid: 50% EBA, 50% eugenol.
L.....	Powder: 69% ZnO, 31% fused silica; liquid: 60% EBA, 38% eugenol, 2% polystyrene
M.....	Powder: 69% ZnO, 31% fused silica; liquid: 40% EBA, 58% eugenol, 2% polystyrene
N.....	Powder: 69% ZnO, 31% fused silica; liquid: 59% EBA, 49% eugenol, 2% polystyrene
O.....	Powder: 68.5% ZnO, 31% fused silica, 0.5% ZnO acetate; liquid: 49% EBA, 49% eugenol, 2% polystyrene
P.....	Powder: 67% ZnO,* 33% fused silica; liquid: 60% EBA, 38% eugenol, 2% polystyrene
Q.....	Powder: 67% ZnO,* 30% fused silica, 3% zinc acetate; liquid: 60% EBA, 38% eugenol, 2% polystyrene
R.....	Powder: 67% ZnO,* 30% fused silica, 3% zinc acetate; liquid: eugenol
S.....	Powder: ZnO; liquid: 50% EBA, 50% eugenol
T.....	Powder: ZnO; liquid: 49% EBA, 49% eugenol, 2% polystyrene
U.....	Powder: 69% ZnO, 31% fused silica; liquid: 50% EBA, 50% eugenol
V.....	Powder: 69% ZnO, 31% fused silica; liquid: 49% EBA, 49% eugenol, 2% polystyrene
W.....	Powder: 90% ZnO, 10% fused silica; liquid: 49% EBA, 49% eugenol, 2% methyl methacrylate
X.....	Powder: 89.7% ZnO, 10% fused silica, 0.3% zinc acetate; liquid: 47.5% EBA, 47.5% eugenol, 5% methyl methacrylate
Y.....	Powder: 90% ZnO, 10% fused silica; liquid: 47.5% EBA, 47.5% eugenol, 5% methyl methacrylate
Z.....	Powder: 89.7% ZnO, 10% fused silica, 0.3% zinc acetate; liquid: 49% EBA, 49% eugenol, 2% methyl methacrylate

* Zinc carbonate was thermally decomposed to zinc oxide for 2 hours at 350° C. ± 10° C. in a nitrogen-gas atmosphere. The oxide was cooled to room temperature in this atmosphere and immediately placed in a sealed container.

number of such mixtures would be theoretically possible. However, the particular formulations chosen were based on the avenues suggested by previous publications in this area and by preliminary screening tests. Mixtures which were impractical from a manipulative standpoint were not included. All the formulations studied may be seen in Table 1; however, the total data will not be presented but only those that are more pertinent and representative. In addition to the additives, it will be noted that certain mixtures involved the use of zinc oxide prepared by the thermal decomposition of zinc carbonate.

All materials were reagent grade. The particle size of the silica was 5 μ.

Mixing was accomplished by placing the weighed amounts of powder and 0.5 ml. of liquid on a chilled glass slab. A stainless-steel spatula was used, and the mix was thoroughly spatulated until no unmixed powder remained.

RESULTS

Compressive strength.—The effects upon the compressive strength of zinc oxide–eugenol by the addition of polystyrene, fused silica, and zinc acetate may be seen in Figure 1. Standard deviations for these tests were below 200 pounds per square inch.

At standard mix consistency, the addition of polystyrene approximately doubled the strength of pure zinc oxide–eugenol. Concentrations above 2 per cent did not increase strength. At this testing consistency, no marked effect on strength could be

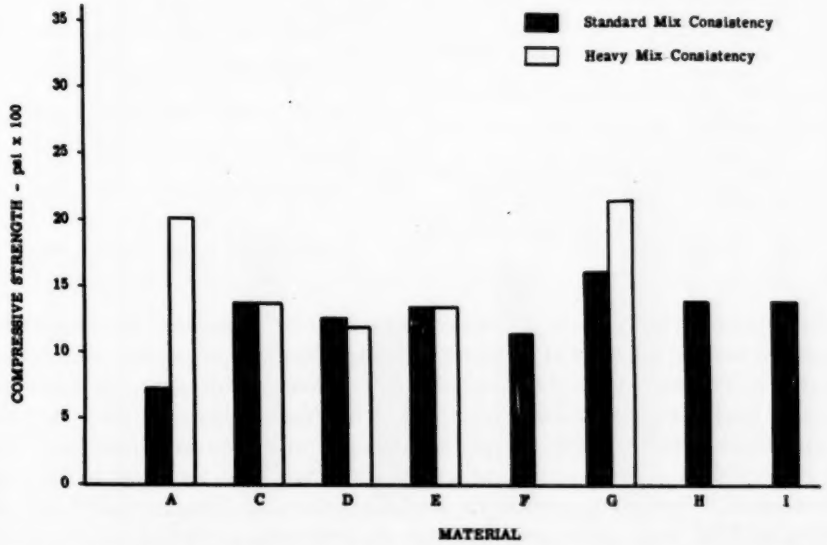

FIG. 1.—Effect of certain additive agents on the strength of zinc oxide–eugenol. Two powder/liquid ratios are shown. *A*—Powder: ZnO; liquid: eugenol. *C*—Powder: ZnO; liquid: 98 per cent eugenol, 2 per cent polystyrene. *D*—Powder: ZnO; liquid: 95 per cent eugenol, 5 per cent polystyrene. *E*—Powder: ZnO; liquid: 90 per cent eugenol, 10 per cent polystyrene. *F*—Powder: 67 per cent ZnO, 30 per cent silica, 3 per cent Zn acetate; liquid: eugenol. *G*—Powder: 67 per cent ZnO, 30 per cent silica; 3 per cent Zn acetate; liquid: 98 per cent eugenol, 2 per cent polystyrene. *H*—Powder: 67 per cent ZnO, 30 per cent silica, 3 per cent Zn acetate; liquid: 95 per cent eugenol, 5 per cent polystyrene. *I*—Powder: 67 per cent ZnO, 30 per cent silica, 3 per cent Zn acetate; liquid: 90 per cent eugenol, 10 per cent polystyrene.

detected by the addition of either silica or zinc acetate. However, when a heavier mix consistency was used (increasing the powder by 50 per cent), the strength did increase for mixture G, where silica had been added. Likewise, with the pure zinc oxide–eugenol material (A) the strength was approximately tripled when a heavier mix was employed. Use of higher powder-liquid ratios for the mixtures where only polystyrene had been added produced strength values comparable to those obtained with the standard mix consistency.

It will be noted that all these values are markedly lower than the strength of zinc phosphate cement, where the minimum strength requirement is 12,000 pounds per square inch. Thus, by comparison, the effect of these particular additives is not significant.

TABLE 2

COMPRESSIVE STRENGTHS OF VARIOUS FORMULAS AT DIFFERENT POWDER/LIQUID RATIOS

FORMULA	MATERIALS	COMPRESSIVE STRENGTH POUNDS PER SQUARE INCH POWDER/LIQUID RATIO IN GM/0.5 ML						
		1.25	1.50	1.75	2.00	2.25	2.50	2.75
M.....	69% ZnO, 31% silica; 40% EBA, 58% eugenol, 2% polystyrene	1,700	1,900	2,300	2,500	2,500	2,800	3,000
O.....	68.5% ZnO, 31 per cent silica, 0.5% zinc acetate; 49% EBA, 49% eugenol, 2% polystyrene	4,600	5,000	5,700	5,900	6,000	.....	.....
S.....	ZnO; 50% EBA, 50% eugenol	.....	4,000	4,400	4,800	5,300	5,500	5,600
T.....	ZnO; 49% EBA, 49% eugenol, 2% polystyrene	.....	5,400	5,800	6,400	6,700	6,800	7,300
U.....	69% ZnO, 31% silica; 50% EBA, 50% eugenol	.....	4,100	4,700	5,300	5,300	5,600	5,800
V.....	69% ZnO, 31% silica; 49% EBA, 49% eugenol, 2% polystyrene	.....	5,100	5,200	5,500	6,200	6,400	6,700
W.....	90% ZnO, 10% silica; 59% EBA, 49% eugenol, 2% methyl methacrylate	.....	5,600	6,000	6,200	6,200	6,900	7,100
X.....	89.7% ZnO, 10% silica, 0.3% zinc acetate; 47.5% EBA, 47.5% eugenol, 5% methyl methacrylate	.....	5,500	5,600	5,900	6,300	6,800	7,300

The influence of EBA (*o*-ethoxybenzoic acid), however, is marked. The compressive strength of varying mixtures of additives of EBA, silica, and polystyrene may be seen in Table 2. The results are shown for different powder-liquid ratios. All these consistencies meet the specification limit of 40 μ for film thickness of zinc phosphate cement. These strength values, except that obtained with formula M, are more than double the highest strength secured at any powder-liquid ratio for mixes of pure zinc oxide–eugenol. These data corroborate the observations of Brauer *et al.*[16] that the addition of EBA does significantly increase the compressive strength of zinc oxide–eugenol. Although the strengths obtained here do not quite reach the 10,000 pounds per square inch reported by those investigators, the formulations differ.

Substitution of equal concentrations of methyl methacrylate for the polystyrene (formulas W and X) did not alter the strength.

It is apparent that the concentration of EBA must be approximately equal to that

of the eugenol, in order to improve the strength. Use of only 40 per cent EBA (mixture M) resulted in strength values less than half those where a comparable mixture (V) was employed but the EBA concentration raised to 49 per cent.

The strength of mixtures prepared with zinc oxide which had been made by the thermal decomposition of zinc carbonate can be seen in Figure 2. A standard mix consistency was used. Although the strength is higher than for a comparable mix of conventional zinc oxide (Fig. 1, mixture *A*), it is still in the same general range. Additive agents did not improve the strength with this type of zinc oxide powder.

Solubility.—In Table 3 can be seen a summary of the solubility of the formulations which demonstrated the highest strength values. The daily solubility and the total at the end of 1 week are shown. Depending on the composition of the specimen, all the mixtures tested, except for the mix of pure zinc oxide–eugenol, are approximately four to eight times more soluble than zinc phosphate cement when tested in the same manner.[18] As might be expected, EBA is apparently the agent primarily responsible for increasing the solubility, although the zinc acetate also raises the solubility slightly.

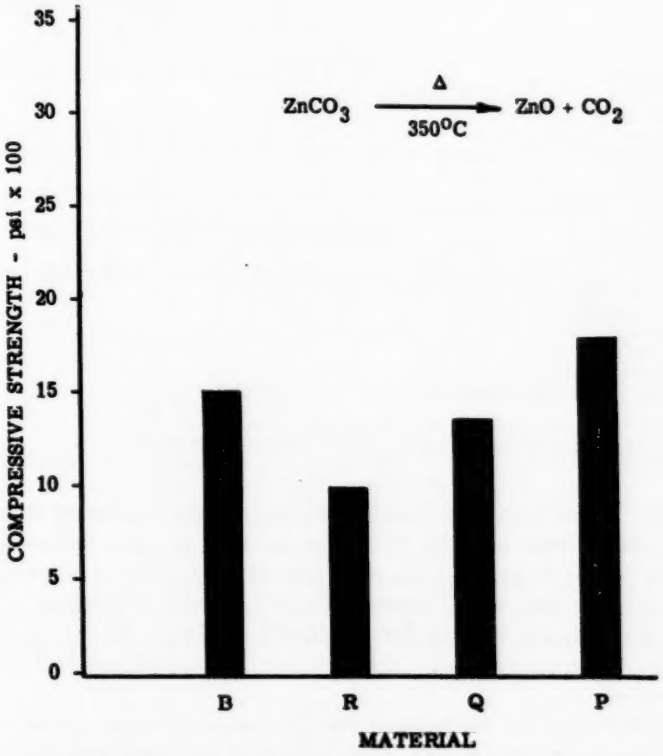

FIG. 2.—Strength of zinc oxide–eugenol mixtures using zinc oxide prepared by thermal decomposition of zinc carbonate. *B*—Powder: ZnO; liquid: eugenol. *R*—Powder: 67 per cent ZnO, 30 per cent silica, 3 per cent Zn acetate; liquid: eugenol. *Q*—Powder: 67 per cent ZnO, 30 per cent silica, 3 per cent Zn acetate; liquid: 60 per cent EBA, 38 per cent eugenol, 2 per cent polystyrene. *P*—Powder: 67 per cent ZnO, 33 per cent silica; liquid: 60 per cent EBA, 38 per cent eugenol, 2 per cent polystyrene.

Addition of polystyrene and methyl methacrylate resins does reduce solubility. However, this is probably due to replacement of the EBA rather than to any true inhibitory action of the resin itself.

The fact that the mixtures tend to undergo the greatest change during the first few days of the test and become relatively insoluble after 5 days may be a somewhat encouraging observation. Zinc phosphate cement[18] and pure zinc oxide–eugenol tend to exhibit essentially a straight-line relationship between solubility and time. Thus it is possible that if the solubility curves of two or three of these least soluble formulations were plotted over a period of time such as a 5-week period, the curves might intersect with those of zinc phosphate cement at that time interval.

TABLE 3

SOLUBILITY OF VARIOUS FORMULAS AT EACH DAY AND TOTAL FOR ONE WEEK

FORMULA	MATERIALS	SOLUBILITY* (PER CENT) TIME IN DAYS							
		1	2	3	4	5	6	7	Total
A.....	ZnO†; eugenol	0.12	0.15	0.08	0.09	0.08	0.11	0.13	0.76
O.....	68.5% ZnO, 31% silica, 0.5% zinc acetate; 49% EBA, 49% eugenol, 2% polystyrene	1.29	0.41	0.27	0.14	0.07	0.02	0.01	2.63
S.....	ZnO; 50% EBA, 50% eugenol	2.03	0.84	0.55	0.38	0.01	0.01	0.00	3.82
T.....	ZnO; 49% EBA, 49% eugenol, 2% polystyrene	1.12	0.73	0.23	0.18	0.02	0.02	0.00	2.30
U.....	69% ZnO, 31% silica; 50% EBA, 50% eugenol	2.20	1.51	0.34	0.24	0.03	0.01	0.00	4.33
V.....	69% ZnO, 31% silica; 49% EBA, 49% eugenol, 2% polystyrene	1.16	0.41	0.27	0.14	0.09	0.03	0.00	2.10
W.....	90% ZnO, 10% silica; 49% EBA, 49% eugenol, 2% methyl methacrylate	1.34	0.82	0.40	0.32	0.04	0.00	0.00	2.93
X.....	90% ZnO, 10% silica, 0.3% zinc acetate; 47.5% EBA, 47.5 eugenol, 5% methyl methacrylate	1.19	0.74	0.41	0.25	0.04	0.00	0.00	2.68

* Solubility and disintegration measured at a ratio of 2.50 gm/0.5 ml. Standard deviation: 0.09%.
† Standard deviation: 0.02%.

Setting time.—Again using the formulas which produced maximum strengths, the setting times can be seen in Table 4. Except for the pure zinc oxide–eugenol and formula Y, the setting times are in the same general range as those of zinc phosphate cement. The working time varied between 3 and 9 minutes, depending on the composition. As expected, zinc acetate does effectively accelerate the set, as does EBA. Generally there was a rather narrow range between the initial and final set of most of these formulas.

Film thickness.—The film thickness of the three mixtures which showed maximum strength may be seen in Table 5. Even though a heavy mix consistency was employed, the film thicknesses are within the A.D.A. Specification No. 8 limit of 40 μ. The addition of fused silica and EBA makes possible the incorporation of two or three times the average amount of powder but with no loss in ability of the cement to be extruded into a thin film.

TABLE 4

SETTING TIME OF SELECTED FORMULAS

Formula	Material	Setting Time (Min.)
A	ZnO; eugenol	10+
O	68.5% ZnO, 31% silica, 0.5% zinc acetate: 49% EBA, 49% eugenol, 2% polystyrene	3
S	ZnO; 50% EBA, 50% eugenol	5
T	ZnO; 49% EBA, 49% eugenol, 2% polystyrene	4
U	69% ZnO, 31% silica; 49% EBA, 49% eugenol, 2% polystyrene	4
W	90% ZnO, 10% silica; 49% EBA, 49% eugenol, 2% polystyrene	9
X	89.7% ZnO, 10% silica, 0.3% zinc acetate; 47.5% EBA, 47.5% eugenol, 5% methyl methacrylate	4
Y	90% ZnO, 10% silica; 47.5% EBA, 47.5% eugenol, 5% methacrylate	10+
Z	89.7% ZnO, 10% silica, 0.3% zinc acetate; 49% EBA, 49% eugenol, 2% methyl methacrylate	3

TABLE 5

FILM THICKNESS OF THREE MIXTURES

Formula	Material*	Film Thickness (μ)
T	ZnO; 49% EBA, 49% eugenol, 2% polystyrene	35
V	69% ZnO, 31% silica; 49% EBA, 49% eugenol, 2% polystyrene	30
X	89.7% ZnO, 10% silica, 3% zinc acetate; 47.5% EBA, 47.5% eugenol, 5% methyl methacrylate	30

* 2.75 gm/0.5 ml mixing consistency.

TABLE 6

IRRITATIONAL CHARACTERISTICS OF THREE FORMULAS WHEN IMBEDDED IN CONNECTIVE TISSUE OF RAT

Formula	Material	Inflammatory Reaction (Days)		
		2	16	32
A	ZnO; eugenol*	Mild	Mild	Mild
O	Zinc phosphate cements†	Mod. Sev.‡	Mod. Sev.	Mod. Sev.
X	68.5% ZnO, 31% silica, 0.5% zinc acetate; 49% EBA, 49% eugenol, 2% polystyrene	Mild	Mild	Mild
	89.7% ZnO, 10% silica, 3% zinc acetate; 47.5% EBA, 47.5% eugenol, 5% methyl methacrylate	Mild	Mild	Mild

* Mitchell (see ref. 1).

† J. B. Boyd, Jr., thesis, Indiana University, 1959.

‡ Mod. Sev. = Moderate to severe reaction.

Irritational characteristics.—The inflammatory reaction of selected formulas to the connective tissue of the rat may be seen in Table 6. All reactions were mild, and the addition of EBA, polystyrene, methyl methacrylate, silica, or zinc acetate did not increase irritation. Compared with zinc phosphate cement, the tissue tolerance is superior.

DISCUSSION

In general, the results of this investigation follow the trends that one might anticipate from previous published data in this field. Although fused silica, polstyrene, and methyl methacrylate may all increase the strength of zinc oxide–eugenol when present in certain concentrations and combinations, the increase is modest. Of the additives studied, only the EBA raised the strength to a value in the lower range of zinc phosphate cements. The exact clinical significance of the high solubility resulting from the addition of EBA is difficult to interpret. Solubility does vary with the test media,[8, 18] and it is possible that in dilute organic acids the difference between the solubility of zinc phosphate cement and zinc oxide–eugenol mixtures containing EBA might not be as great. At any rate, the higher solubility induced by the EBA is not desirable, and further research should be devoted to the use of other agents which would be less soluble but which might still produce a comparable chelating reaction.

A word should be said concerning certain factors that apparently influence the handling characteristics of these materials. Although fused silica does not greatly influence strength, it did produce a smoother, more easily spatulated mass and must be in concentrations of at least 10 per cent in the powder. The addition of polystyrene produces some difficulty, as it tends, in time, to precipitate from the solution. Although heating the liquid will usually cause the precipitate to redissolve, substitution of methyl methacrylate powder produced greater stability of the solution and gave comparable physical properties.

Although the use of zinc oxide prepared by the thermal decomposition of zinc carbonate did not increase the strength of the formulas studied, it did produce a rapid setting and non-tacky mixture which might eventually be developed as a temporary restorative material.

SUMMARY

A study was made on the effect of selected additive agents upon certain properties of zinc oxide and eugenol.

Addition of *o*-ethoxybenzoic acid (EBA), if in approximately equal parts with eugenol, produced significant increases in compressive strength. It simultaneously increased solubility and decreased the setting time. Solubility also increased slightly by the addition of silica and zinc acetate. Addition of polystyrene was more effective in increasing the strength when present in mixtures containing EBA.

Zinc oxide produced from the thermal decomposition of zinc carbonate did not increase the strength of the mixtures studied but did decrease the setting time and tackiness of the mix.

Although not markedly altering strength, the addition of fused silica improved the handling characteristics of the mixtures when in a concentration of at least 10 per cent in the powder.

Substitution of methyl methacrylate for polystryrene increased the stability of the

solution and had an effect comparable to the polystyrene on strength and solubility.

Inflammatory reactions in the connective tissue of the rat were mild for all mixtures tested.

Zinc acetate effectively accelerated the set of all mixtures.

The exact effect of agents added to mixes of zinc oxide and eugenol depends on the particular combination employed and the property being evaluated. Further research may make possible materials of this type having physical properties comparable or superior to zinc phosphate cements.

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Cytochemical and Biochemical Studies of Aerobic Oxidative Metabolism of Human Gingiva

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When mammalian tissues are grouped according to their respiratory or oxygen-utilizing capacities, gingiva is one of a group characterized by relatively low oxygen consumption. A representative Q_{O_2} value (dry-weight basis) for slices from slightly inflamed human gingiva is 1.6.[1] Other tissues in this category, together with typical Q_{O_2} values are skin (human), 1.6–1.8;[2] rib cartilage (rat), 0.22–0.66;[3] cornea (rabbit), 0.46–2.09,[4] and lens (rabbit), 0.05–0.21.[5] It has been shown that the endogenous oxygen uptake of human gingiva is highest during the first 10 minutes of manometric determinations and declines rapidly with time, so that, if measurements are based on the first 10-minute interval, somewhat higher endogenous respiration rates are obtained.[6] Notwithstanding attention to such details, reference to Q_{O_2} values of active oxygen-consuming tissues, e.g., 15 in the case of rat kidney,[7] readily illustrates the wide disparity in the rates of aerobic metabolism of the different tissue types. With reference to other studies of gingival respiration,[8-10] we found only one report in which Q_{O_2} values appreciably higher than those mentioned above were reported. Leng listed values as high as 10.65.[10] We know of no other instance in which such high Q_{O_2} values have been obtained and can offer no interpretation of this discrepancy.

The above studies have dealt primarily with measurements of over-all oxygen utilization by gingival tissue slices and homogenates. Most recently, Eichel reported preliminary observations on six oxidative enzyme systems of human gingiva, using spectrophotometric and histochemical methods.[11] His data for cytochrome oxidase activity of human gingiva as determined by spectrophotometric assay (while not directly comparable) were in accord with those of the above-mentioned manometric studies (excepting the paper by Leng).

Since there had been so few studies of intermediary metabolic processes involved in the respiration of human gingiva, the present investigation was undertaken. In this paper we have evaluated the relative oxygen-utilization capacities of the different cell layers and cell types of gingiva, using the synthesis of indophenol blue from α-naphthol and dimethylparaphenylenediamine by fresh frozen slices of human tissue, i.e., the "G" Nadi reaction.[12-14] We shall also present correlated measurements of the cytochrome oxidase activity of gingival homogenates as determined by a standard manometric method.[17]

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MATERIALS AND METHODS

Gingival specimens were obtained in the course of periodontal surgery from twenty-five male patients ranging between twenty-four and fifty-two years of age. Anesthesia was produced only by regional nerve block. Immediately following excision, tissue specimens were rinsed with cold distilled water and placed in a moist, covered Petri dish set in cracked ice. The clinical condition of the gingivae varied from normal to slightly inflamed; no visibly necrotic tissue was used. For the cytochemical experiments, frozen sections were cut at 20 or 40 μ thickness. For the manometric experiments, tissue was cut at 100–200 μ thickness. The latter slices were then homogenized in 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 6.9, using a ground-glass tissue homogenizer.

Dimethylparaphenylenediamine • 2HCl was prepared from the free base according to the method of Michaelis, Schubert, and Granick.[15] α-Naphthol was purified by sublimation. Both reagents were prepared freshly before each experiment as 0.01 M solutions. The incubation medium consisted of 1.0 ml. of each reagent mixed immediately prior to use and added to 2.0 ml. of the PO_4 buffer. (This mixture will henceforth be referred to as the "reagent mixture.") The frozen sections were floated directly into the reagent mixture (contained in a Syracuse dish), and color development was followed with a microscope. In the inhibitor experiments, azide and cyanide were incorporated into the above mixture, to yield final concentrations ranging from 10^{-2} to 10^{-5} M. Experiments were also performed with urethane in the same range of final concentrations. The purpose of the urethane was to inhibit reducing systems which compete with the "G" Nadi reaction.[16] Several experiments were also performed, in which CO (20 per cent CO, 80 per cent air) was used as an inhibitor. In all experiments, alternate frozen sections were fixed in 10 per cent formalin and stained with hematoxylin and eosin for reference purposes.

A standard manometric procedure for cytochrome oxidase[17] was used. When sufficient tissue was available, determinations were made in duplicate; if not, single vessels were run. Controls for auto-oxidation of substrate (hydroquinone) accompanied each determination. Boiled tissue homogenates were completely negative. Values were expressed as Q_{O_2} per mg. N of homogenates. Nitrogen determinations were made by a modified Nesslerization technique.[18]

RESULTS

The time sequence of indophenol blue synthesis was followed in most experiments. Blue color first appeared in the basal-cell layers of the crevicular epithelium, usually 3–5 minutes following incubation of slices. Color formation was next apparent in the basal-cell layers of the attached gingivae. Following the appearance of color in the above sites, there was a slow spread to the prickle-cell layers above the basal cells. This usually required 20–30 minutes. After approximately 60 minutes of incubation, the outermost layers of the tissue showed color development. Most interesting was the observation, 60–90 minutes following incubation, that both keratin and parakeratin developed a deep-blue coloration.

In Figure 1, a section of gingiva is shown following 90 minutes of incubation. Note the deep coloration of the outer keratin layer. In Figure 2 we see a higher power of the section shown in Figure 1. The deep-blue stain of the keratin layers is quite evident;

however, the presence of pyknotic nuclei indicates that parakeratin and not true keratin was present. A similar intense blue coloration was also found in those cases in which true keratin was present. Note also in Figure 2 that colorless oval areas are to be seen throughout the section. These represent nuclei, in which indophenol blue synthesis does not occur. It will be recalled that the sites of aerobic oxidation are in the cytoplasmic mitochondria.[19] For this reason, in the sections studied, nuclei appeared as colorless ovoid areas, while the insoluble granules of indophenol blue were formed and remained in the surrounding cytopasm. For reference orientation, the reader's attention is directed to Figure 3, a serial section made from the same tissue, fixed in 10 per cent formalin and stained with hematoxylin and eosin.

Both cyanide and azide inhibited indophenol blue synthesis by gingival slices, as seen in Figure 4: 10^{-2} M inhibited completely, 10^{-3} M was strongly inhibitory, 10^{-4} M less so, and 10^{-5} M only slightly inhibitory. Quantitation of the inhibitions was not attempted because, in contrast to most other tissues from which indophenol blue can be dissolved by ethyl alcohol or similar solvents, it was found that the dye was so tightly bound to both parakeratin and keratin that all solvents used thus far could not leach it out.

The foregoing data indicated that the enzyme responsible for indophenol blue synthesis *might* be cytochrome oxidase. For this reason experiments were performed in which tissue sections were incubated in a Nadi reagent mixture gassed with 20 per cent CO–80 per cent air prior to and during the incubation. Of five such experiments, in two instances a light-reversible inhibition of indophenol blue synthesis was observed. However, this was not reproducible in the other experiments of the series. Thus it is not possible to say with certainty that the indophenol blue synthesis by the sections was mediated specifically via the enzyme cytochrome oxidase.

It is known that indophenol blue synthesis can be inhibited by the presence in cells of active anaerobic dehydrogenases which compete for electrons with aerobic systems whose oxidations are mediated via oxygen.[16] Since urethane is an inhibitor of such dehydrogenases, several experiments were performed in which urethane, in the range 10^{-2} M–10^{-5} M was incorporatd into the reagent mixture. Results were extremely variable. In some instances there appeared to be an enhanced synthesis of indophenol blue. In other instances urethane was completely without effect. This may have been due to different levels of endogenous dehydrogenase and substrates in different tissue samples. It is planned to continue study of this aspect of the problem in future experiments.

Using hydroquinone as substrate, in the presence of added cytochrome *c* the cytochrome oxidase activity of most gingival homogenates, expressed in terms of Q_{O_2} (N), ranged between 17 and 25. When converted to Q_{O_2} (dry weight), these give values between 2.7 and 4.0, which are slightly higher than endogenous respiration rates. However, since in the present assay excess substrate is added to a limiting enzyme concentration, higher values than those obtained for endogenous respiration are to be expected. In one experiment, not included in the above range of values, a Q_{O_2} (N) of 6 was obtained. In several experiments, homogenates were completely inactive.

Study of the oxygen-uptake values in different experiments is of great interest. In Figure 5, oxygen utilizations versus time are shown. All values are corrected for auto-

FIG 1.—Fresh frozen section of human gingiva incubated in "G" Nadi reagent mixture and demonstrating pattern of indophenol blue synthesis after 90 minutes' incubation. The sections shown in Figures 2, 3, and 4 are serial sections made from the same tissue specimen.

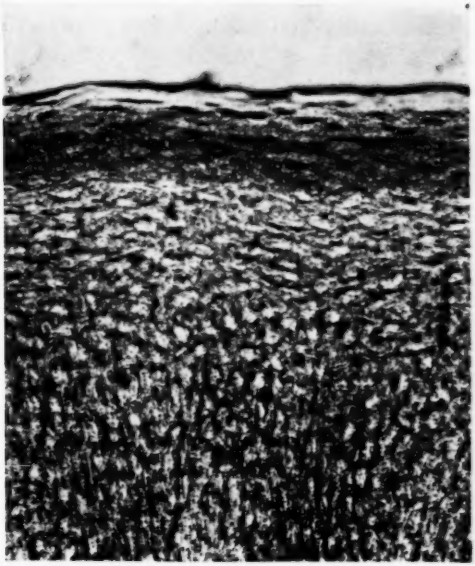

FIG. 2.—Higher-power view of epithelium of section shown in Figure 1, demonstrating presence of indophenol blue in parakeratin close to upper border of the section.

FIG. 3.—Serial fresh frozen section of human gingiva (hematoxylin-eosin stain) for reference.

FIG. 4.—Control; serial fresh frozen section of human gingiva incubated in reagent mixture containing $10^{-3}M$ cyanide to inhibit indophenol blue synthesis. The increased optical density of the subepithelial connective tissues does not represent dye synthesis. This effect appeared to be a result of cyanide treatment.

oxidation of substrate. In curve *1* it may be seen that, after an initial lag of 30 minutes, oxygen consumption began and then came to a halt after 120 minutes. In curve *2*, an initial lag was not present, oxygen utilization began immediately but ceased after 60 minutes. In curve *3* the activity stopped after 90 minutes. This type of variability was most interesting because we had encountered similar phenomena during a study of cytochrome oxidase activity of cartilage.[20, 21] In the case of cartilage it has been possible to demonstrate the presence of endogenous substances within the tissue, which were inhibitory to cytochrome oxidase activity. To test this possibility in regard to gingiva, experiments were performed in which gingival homogenates were added to very active beef-heart muscle cytochrome oxidase preparations.[22] In half-a-dozen separate experiments of this kind, the gingival homogenates were inhibitory on three

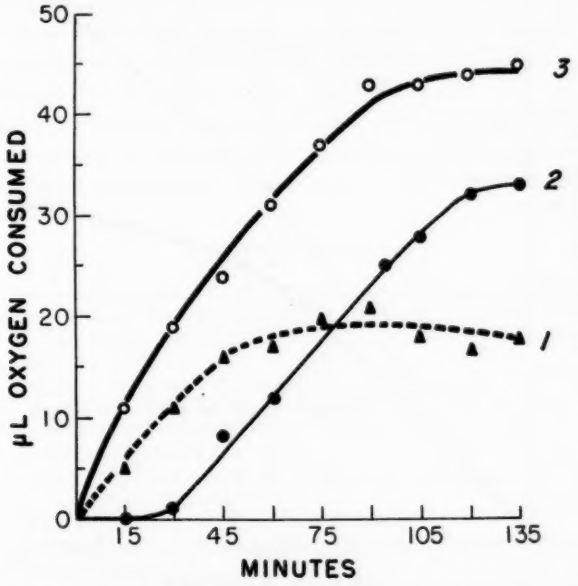

FIG. 5.—Manometric estimation of cytochrome oxidase activity of human gingiva. Illustrates variable nature of oxygen consumption by homogenates from different specimens of tissue.

occasions. A typical experiment is shown in Figure 6, demonstrating that addition of human gingival homogenate to an active heart-muscle cytochrome oxidase preparation resulted in decreased oxygen utilization by the oxidase.

DISCUSSION

Several of the observations made during these studies warrant further discussion. The appearance of indophenol blue in the parakeratin and keratin outer layers of gingiva is of great interest. Three possibilities (not necessarily mutually exclusive) have been considered in relation to this phenomenon. First, on standing, certain groups within the keratins may be altered or unmasked, thereby conferring upon the structures a higher oxidation potential. One thinks of disulfide-sulfhydryl equilibria in this

context. Second, the indophenol blue formed via auto-oxidation of the Nadi reagents may be selectively dissolved by lipids in the keratins. Third, keratin may act as a semiconductor and may thereby be involved in an electron transport capable of mediating oxidation of the reagents. This latter possibility arises from a consideration of the work of Szent-Györgi,[23] who, on the basis of quantum reasoning, has postulated semiconductor properties for proteins, and also from the work of King and Melley,[24] who demonstrated and measured ionic conductances of keratin. In view of the above, we may consider the possibility that, far from being an inert protective covering, keratin may possess oxidation-reduction (and other metabolic) properties which, although low,

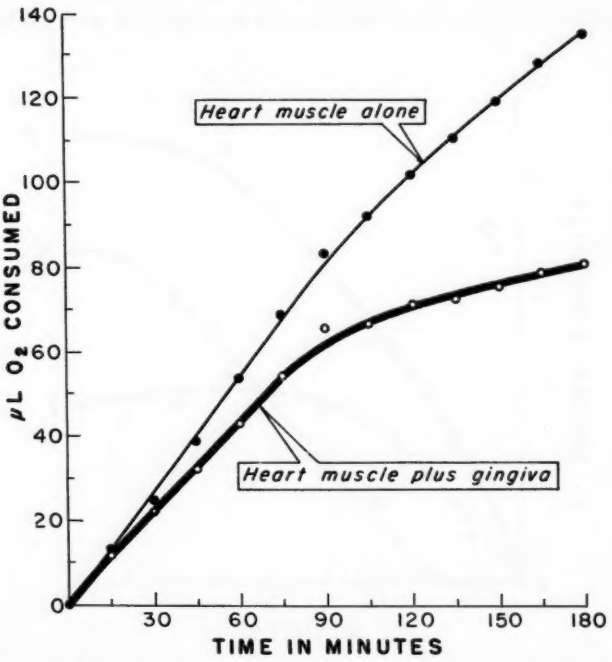

FIG. 6.—Inhibition of heart-muscle cytochrome oxidase activity by added gingival homogenate

may be of considerable significance. The strong binding of the indophenol blue to the keratin layers is also worthy of note and can be a means of determining the nature of the available binding sites within the tissue.

The last experiments reported deal with the inhibition of heart-muscle cytochrome oxidase activity by gingival homogenates. Although these are only beginning observations, they are considered significant. It may well be that a part of the explanation for the low aerobic oxidative activity of gingiva lies in the presence within the tissue of substances which are inhibitory toward the respiratory enzymes. The inhibition of cytochrome oxidase activity by polyonic components of cells and tissues has been demonstrated by Smith and Conrad, who reported that protamine, a polycationic constituent of cell nuclei, was inhibitory to active oxidase preparations.[25] More recently we have discovered a greater number of such inhibitions that are *reversible* by the

addition to anionic macromolecules.[26] Thus the regulation of cell respiration by endogenous cell components becomes an important consideration, especially when slice and homogenate techniques are used. Further studies along these lines are in progress in our laboratory.

SUMMARY

A correlated cytochemical and biochemical study has been made of aerobic oxidase activity of human gingival slices and homogenates. Indophenol blue synthesis by fresh-frozen sections was used as a means of assessing relative oxidation capacities of different cell types of the gingival epithelium. Crevicular basal cells were the first to synthesize the dye, followed by basal cells of the attached gingiva. Color development then proceeded outward to surface layers of the tissue. Parakeratin and keratin stained deeply after 60–90 minutes' incubation in the reagent mixture. Indophenol blue could not be leached out of the keratin or parakeratin by a large number of solvents used for this purpose with other tissues. In manometric assays of cytochrome oxidase activity of gingival homogenates, Q_{O_2} (N) values between 17 and 25 were obtained, corresponding to Q_{O_2} (dry-weight) values of 2.7–4.0. Some preparations were completely inactive manometrically. In beginning experiments, addition of gingival homogenates to active heart-muscle cytochrome oxidase preparations inhibited activity of the oxidase.

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Ultrafilterable Calcium in Caries-susceptible and Caries-immune Subjects

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The initiation and propagation of the carious lesion depend on the concentration of hydrogen calcium and phosphate ions in the fluid immediately overlying the enamel.[1] Among the factors influencing the concentration of calcium ions in the immediate vicinity of the lesion is the extent to which salivary calcium is bound to salivary protein. Such binding is the subject of notes by Krasnow[2, 3] and is considered in the work of Schmidt-Nielsen.[4]

In the following is described the measurement of ultrafilterable calcium in the saliva of twenty subjects, half of whom appear to be very susceptible and half much less susceptible to caries. The measurement has been made at two pH values for each saliva: at the pH of the saliva as collected and at a more acid pH between 4.5 and 6.5.

EXPERIMENTAL METHODS

The samples were obtained with paraffin stimulation from patients of F. C. B., all of whom had been under observation a number of years. About 20 ml. of saliva from each subject were mixed with a few drops of toluene and placed in the refrigerator until just before analysis. On removal, the pH was measured, the saliva was centrifuged, and samples of the supernatant were taken for calcium analysis.* About 4 ml. of the supernatant was placed in each of two stoppered pieces of Visking $\frac{3}{8}$-inch cellophane tubing which was suspended in a 15-ml. centrifuge tube and centrifuged at 3000 r.p.m. for about 45 minutes. Somewhat over 0.5 ml. of ultrafiltrate was obtained in each centrifuge tube, and 0.500 ml. was analyzed for calcium. The analyses of calcium in supernatant saliva and ultrafiltrate were done with ashing by the method of Harnach.[5] The remaining supernatant of each sample was mixed with sufficient 1N acetic acid to change the pH to from 4.5 to 6.5 (for half between 5.5 and 5.9). The acidified salivas were subjected to ultrafiltration in the same way, and the ultrafiltrate was analyzed for calcium.

The presence of colloidal calcium phosphate and the effect of the method of collection on the formation of colloidal calcium phosphate were investigated by centrifugation at high speeds, as done by Levinskas.[6]

Two saliva samples were collected from each of three subjects; one under oil into a centrifuge tube by means of a narrow polyethylene tube and the other with toluene

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* In preliminary experiments it was shown that these procedures did not change the pH of the saliva from its original value.

exactly in the same way as the samples from patients. The samples were all placed in the refrigerator at 0° C. for 5 days, centrifuged to bring down sediment, and analyzed for calcium. The drop in calcium represents the colloidal calcium phosphate if this acts as in Levinskas' solutions. This drop amounted in the specimens collected under oil to 1, 2, and 1 per cent and in the specimens not collected under oil to 16, 3, and 3 per cent. It was concluded that, under the circumstances of the experiment, the non-ultrafilterable calcium is chiefly protein-bound and not colloidal.

RESULTS

The results given in Table 1 show that the ultrafilterable calcium constitutes from about 40 per cent to about 80 per cent of the total calcium when the pH of the saliva

TABLE 1

Subject No.	pH	Total Calcium (Mg. Per Cent)	Diffusible Calcium (Per Cent of Total)	Clinical Observations			
				Age	Sex	DMF	Remarks
1.....	7.7	4.04	68	14	F	12/28	Active
2.....	7.6	3.84	65	21	F	20/30	Active
4.....	7.6	4.76	75	19	M	12/30	Active
5.....	7.7	3.68	84	26	F	18/30	Active
13.....	7.5	5.12	68	19	F	20/30	Active
14.....	8.1	3.04	62	9	F	14/24	Active
15.....	7.6	4.92	69	27	F	23/32	Active
16.....	7.8	4.28	66	19	F	25/32	Active
17.....	8.0	4.56	47	15	F	14/28	Active
19.....	7.8	4.72	43	15	M	17/28	Active
3.....	7.6	3.56	73	21	F	1/32	? One active area
6.....	7.8	3.16	75	11	F	0/28	
7.....	7.5	5.12	72	17	M	8/28	Preventive fillings
8.....	7.6	4.84	65	45	F	10/28	Inactive 5 years
9.....	7.9	5.16	60	16	M	0/28	
10.....	7.5	5.24	64	18	M	2/28	Two small pits
11.....	7.6	4.60	71	25	F	0/32	
12.....	7.8	4.24	78	30	F	6/28	Inactive 10 years
18.....	8.0	2.44	67	15	F	0/28	
20.....	7.9	3.88	44	13	M	0/28	

lies between 7.5 and 8.1. There does not appear to be any correlation between caries activity as observed clinically and the fraction of the calcium which is ultrafilterable. The average percentage of the total calcium which is ultrafilterable is the same in saliva from clinically very active and from clinically inactive subjects.

The ultrafilterable calcium in the same salivas acidified to pH values between 4.5 and 6.5 constituted between 92 and 106 per cent of the total, and in the three most slightly acidified salivas (pH 6.3, 6.3, 6.5) and in three-quarters of all the acidified salivas it lay within 3 per cent of the total.

SUMMARY

About 40–80 per cent of the calcium in saliva is ultrafilterable at normal alkaline pH values, but all the calcium becomes ultrafilterable when the saliva is made slightly acid.

In twenty subjects, half with active clinical caries and half with little or no caries

activity, there was not observed any relation between the percentage of the total calcium which was ultrafilterable and the caries activity.

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The Pathways of the Early Carious Process
I. Histologic Study

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There are generally said to be three components of the structural unit of enamel: the interprismatic substance, a continuous phase which in cross-section looks like a net; a separate phase, the rods, which have rather complex cross-sections; and a third phase, the sheath, which more or less completely covers each prism and is accordingly seen in cross-sections as a more or less complete ring around each rod. The sheath, after the early stages of histogenesis, is space rather than membrane and becomes smaller and smaller as the calcification of the erupted tooth reaches completion.[1–3] Statements of the changes in these three elements as the enamel becomes carious have been conflicting. In the recent literature numerous investigators have suggested that the interprismatic substance was preferentially removed by the acid of caries, leaving the rod material undissolved until later.[4–6] These reports are at variance, however, with older investigations[7, 8] which suggest that the sheath or the rod itself is attacked first.

In the study described here it is shown that the sheath is the first component of the enamel to become involved in the carious process and the cross-striations and the bodies of the prisms next and that this order of attack has great significance for the nature of the carious process.

MATERIALS AND METHODS

Thirteen permanent teeth with early white-spot caries and one unerupted third molar were examined. Longitudinal sections 200 μ thick were cut in series from each tooth, so that two or more sections of each lesion were obtained. There were no lesions in the unerupted tooth, and only one section near the center was chosen for study. Each longitudinal section was marked by means of small holes drilled in the dentin and by a thin metal-foil strip glued near the lesion. Photographs were made in polarized light after the lesions were marked. After this a transverse cut was made through the longitudinal section in the region of the lesion, and another photograph was taken. In this way a tracing of the second photograph could be superimposed on the first, properly oriented by means of the markers, and a line drawn showing the precise level at which the transverse section was cut from the longitudinal section (see Figs. 1 and 2). Furthermore, since a portion of the metal-foil marker appeared on the transverse section, the distance from it of any field studied under the microscope could be ascertained, and this field located on the photograph of the longitudinal section.

The transverse sections were prepared by gluing the 200-μ longitudinal sections to a

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lucite backing piece, fastening this securely to a metal holder on the cutting machine, and cutting a section transverse to the rods with a diamond wheel. In this manner 80-μ transverse sections were cut from the longitudinal sections, resulting in sections 80 μ thick and 200 μ wide.

The transverse sections were impregnated with mercury sulfide according to the technique described by Nishimura.[9] A few sections were stained also with ammoniacal silver nitrate according to the method of Howe.[10] Stained and unstained sections were viewed in ordinary transmitted light.

RESULTS

Various investigators have shown that a translucent appearance of enamel is the earliest recognizable stage of caries.[5, 9, 11] In serial sections through lesions we found that the first and last sections showed superficial carious areas which were largely

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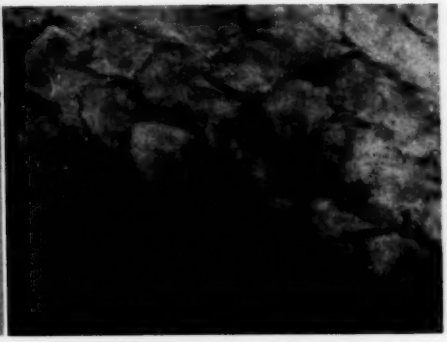

1*A*

FIG. 1.—Photomicrograph of a 200-μ serial section near the edge of a carious lesion. The enamel is largely translucent in appearance. The line designates the level at which a transverse cut was made through the carious area. Copied from a color slide made in polarized light with a Red I Plate. 10 ×.

FIG. 1*A*.—Photomicrograph of an 80-μ transverse section made through the level designated by the line in Fig. 1 and impregnated with mercury sulfide. This figure represents the transverse appearance of the rods in the region marked *A* in Fig. 1. Note the dark granular impregnation at the periphery of the rod while the prism bodies are as yet unstained. 1,200 ×.

translucent in reflected light (Fig. 1). Staining occurred in such areas only at the outer rim of the prisms. Figure 1*A* is a photomicrograph of the impregnated prisms in region *A* of Figure 1. Here one can see that only the prism periphery has been impregnated. Serial longitudinal sections near the center of the lesion showed carious areas which penetrated more deeply into the enamel and consisted of large regions of positive birefringence with a narrow translucent zone at the periphery (Fig. 2). Figures 2*A* and 2*C* represent areas *A* and *C* at the periphery of the lesion in Figure 2. In both cases there is very little staining of the prisms. Only the outer rims are darkly stained. In more advanced regions the impregnation increased in intensity in the prism body from the periphery toward the center until the entire body was heavily impregnated. Figure 2*B*, from a centrally located field in the carious area of Figure 2, illustrates this. The interprismatic substance, however, is only lightly stained at this stage. It is evident from this photograph that the early impregnation at the periphery of the prism lies *between* the prism body and the interprismatic substance.

In many areas where the prism bodies were well stained, each prism was seen to have one or more processes extending between neighboring prisms (Fig. 3). Such processes were noted in enamel by Smreker[3] and von Ebner[12] and termed "alar processes" by the former. Scott[13] more recently has also reported similar enamel processes found in electron-microscope replicas and sections. In unstained sections these alar processes may easily be mistaken for interprismatic substance—especially in longitudinal sections—and may account for the interpretation by some authors that the interprismatic substance becomes markedly demineralized at an early stage.

Sections from an unerupted third molar were stained by the mercury impregnation technique also. Figure 4 is a photograph of one such section, indicating the level at which the transverse cut was made. Figure 5 shows a stained area from the transverse section. Here one can see that impregnation occurs at the outer edge of the prism. In no case was sound enamel of posteruptive teeth found to stain with the mercury sulfide impregnation technique. This would seem to indicate that the region at the periphery of the prism is the last area to calcify in development, as well as the first to decalcify in

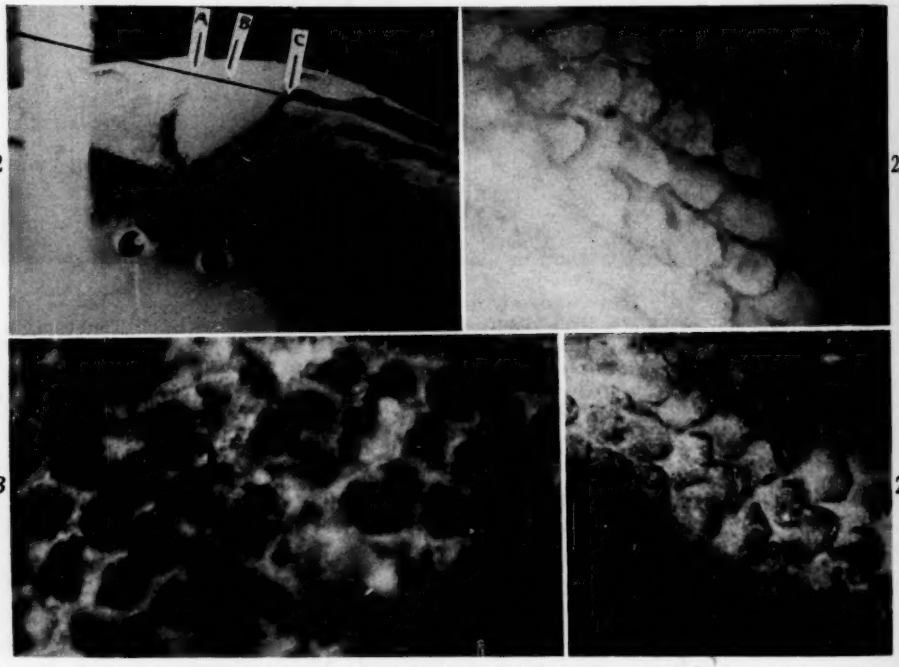

FIG. 2.—Photomicrograph of a 200-μ serial section near the center of a carious lesion—a more advanced area of the lesion than the section in Fig. 1. Both sections are from the same lesion. The line designates the level at which a transverse cut was made through the carious area. Copied from a color slide made in polarized light with a Red I Plate. 10 ×.

FIG. 2*A, B, C*.—Photomicrographs of an 80-μ transverse section made at the level designated in Fig. 2 and impregnated with mercury sulfide. Figure 2*A* represents the transverse appearance of the rods in the region marked *A* in Fig. 2; 2*B* the rods in the region marked *B*; and 2*C* the rods in the region marked *C*. In Figures 2*A* and 2*C* at the translucent edge of the carious area, dark impregnation occurs at the periphery of the prism, while the prism bodies show very little. Figure 2*B*, from the center of the carious area, shows that the entire prism has become impregnated, while the interprismatic substance has not. 900 ×.

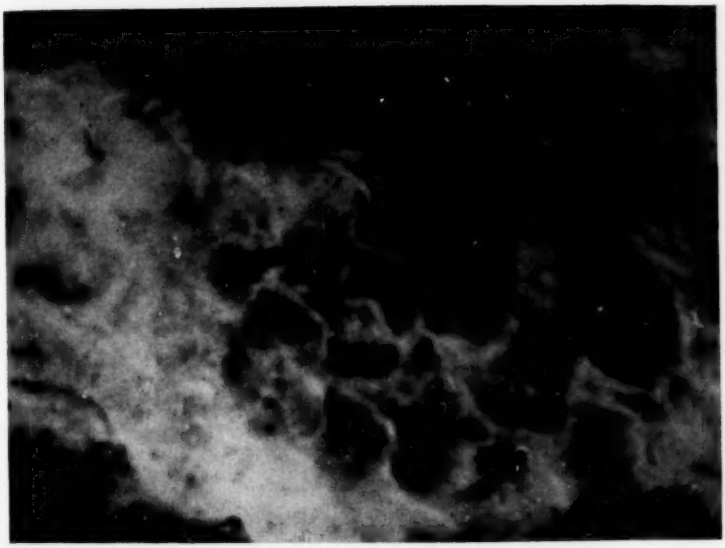

FIG. 3.—Photomicrograph of an 80-μ transverse section near the center of a carious area. Here the prism bodies are completely impregnated, as in Fig. 2*B*, but each prism is seen to have a process extending between adjacent prisms. Note also the unimpregnated interprismatic substance which is more or less arcade-shaped. 2,000 ×.

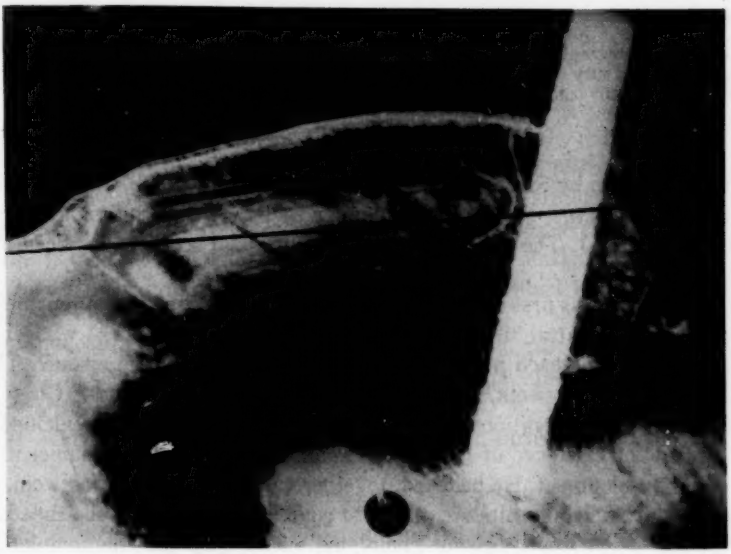

FIG. 4.—Photomicrograph of a 200-μ longitudinal section through an unerupted third molar. The line designates the level at which a transverse cut was obtained through this section. Copied from color slide made in polarized light with a Red I Plate. 17 ×.

caries. It was found, however, that the region at the periphery of the prism could be stained in occasional areas of posteruptive teeth with ammoniacal silver nitrate. Sullivan felt that his studies with silver nitrate demonstrated that the sheath of sound enamel was permeable to small molecules, and he believed that this implicated it as a pathway of acid in caries.[8] In general, the observations made in this study agree with those of Nishimura, although our interpretation, as seen in the discussion, differs somewhat.

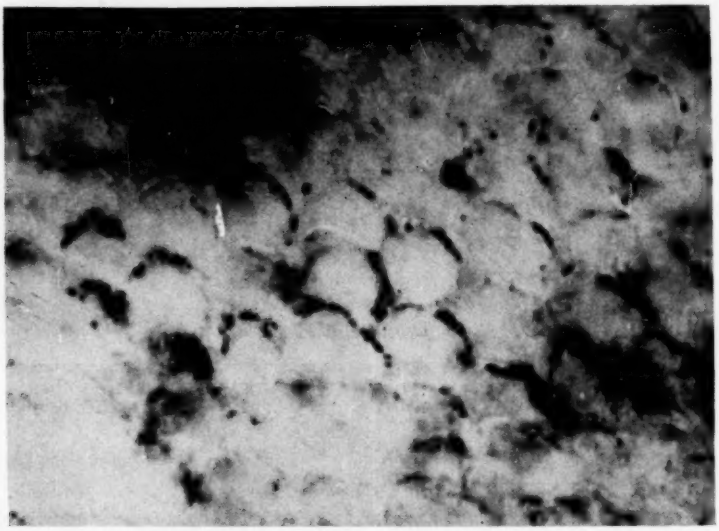

FIG. 5.—Photomicrograph of an 80-μ transverse section made at the level designated in Fig. 4 and impregnated with mercury sulfide. Only the periphery of the prism has become impregnated, indicating that it is not yet fully calcified. 1,500 ×.

DISCUSSION

As was first shown by Smreker[3] and confirmed by von Ebner,[12] Chase,[1] and Scott,[13] the enamel prism in cross-section has a complex shape which includes one or more alar processes (see Fig. 3). The interpretation of longitudinal sections has been extremely difficult.

The prism sheath was first described by Smreker[3] in cross-sections of enamel impregnated with acid or neutral silver nitrate and named by Studnicka,[14] who observed a membrane surrounding each prism early in the histogenesis of the enamel. The prism sheaths, according to Jasswoin,[2] "appear as stained crevice-like spaces." Chase,[1] on the basis of extensive observations on serially sectioned, partially decalcified human enamel, denied the existence of the sheath as a stainable entity in enamel which had completed its development. He believed, together with Smreker and Jasswoin, that the pathway by which calcium salts are added to enamel might well be the sheaths, which "when mineralization would become completed would then become completely calcified." The sheath in enamel beyond the early stages of development thus appears to be space and pathway rather than organic structure. At any stage it lies between the prism and the interprismatic substance.

There can be little doubt that the sheath space actually does serve as a pathway for the calcification of the enamel. It communicates with both the pulp and the exterior of the enamel to varying extents at various stages in enamel dvelopment. It is the only structure sufficiently permeable to account for the transport of material in calcification in the amounts which actually occur, and it is known to have its walls increasingly calcified in the last stages of enamel calcification.

In Nishimura's impregnation method, black mercury sulfide is deposited in the spaces which have been filled with gelatin. That the spaces impregnated by mercury sulfide in the cross-sections thrcugh the earliest areas of caries are the reopened sheath spaces is certain for a number of reasons. The location of the impregnated areas between the prism and the interprismatic substance is the location of the sheath space. Moreover, the greater susceptibility of this area to solution is readily understood. As minerals are deposited in the sheath spaces, eventually filling them and progressively reducing the flow through the sheath space, it is to be expected that the most insoluble material will be deposited first and the most soluble material last. The area in which the last material is deposited should accordingly be the area first dissolved by the carious process.

That the first step of the carious process reverses the last step of enamel calcification is important because this reversal opens up a pathway connected with submicroscopic channels deep in the enamel. The opening of these channels in the cross-striations and in the bodies of the prisms can be followed early in caries with the polarizing microscope. It is its reversal of the calcification of enamel that gives the early carious lesion its penetration and subtly discriminating leaching ability—the characteristic from which it derives its name "caries," the Latin term for dry rot. The gross, the microscopic, and the submicroscopic phenomena are thus connected with the nature of enamel development and calcification.

SUMMARY

A study was made of transverse sections taken from early and advanced carious zones and impregnated by the mercury sulfide technique of Nishimura. Impregnated sections from non-carious unerupted third molars were also studied.

The earliest impregnation was seen at the periphery of the rod in the sheath, indicating that this is the first site of change in caries. In more advanced areas the prism body became progressively impregnated from its periphery toward its center, leaving the interprismatic substance unstained.

The sheath was found to impregnate with mercury sulfide in an unerupted third molar but not in sound enamel of posteruptive teeth, indicating that it does not calcify completely until very late in development.

The significance of the sheath in development and in caries is discussed.

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The Pathways of the Early Carious Process
II. Radiologic Study

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The preceding paper has served to demonstrate the sites of early carious changes. However, it is worthwhile to demonstrate that these changes are indeed due to decalcification of enamel material. Numerous reports have appeared dealing with microradiographic studies of beginning caries.[1–4] However, few of these have attempted to deal with the high-resolution aspects of lesions and with the microradiographic appearance of transverse sections. This study was prompted by the belief that certain of the morphologic details are more easily understood in transverse section and that high-resolution study of such sections, together with longitudinal sections of lesions, might aid in clearing up some of the existing uncertainties with regard to the site of the early carious decalcification.

MATERIALS AND METHODS

Ground sections were prepared from 24 permanent teeth with early white-spot smooth-surface lesions. The teeth were imbedded in plastic cylinders* and sectioned longitudinally at 250-μ thickness on a cutting machine. They were then glued to plastic cover slips and ground to a thickness of 20–60 μ. After grinding, the cover-slip backing was dissolved from the section with dichloroethane, and that portion of the section containing the lesion was mounted on a thin sheet of cellulose acetate which had been stretched across a metal ring designed to fit into the X-ray machine. Microradiographs were made of both longitudinal and transverse sections. The transverse sections were prepared as described above, except that the 250-μ section first cut from the tooth was examined for a lesion, then reimbedded in plastic, and a transverse cut made through the enamel rods in the area of the lesion. It was found, especially in the case of thicker sections, that staining with aldehyde fuchsin before X-ray resulted in better detail in the X-ray picture. This was believed to be due to the fact that the fuchsin was dissolved in acid alcohol, which appeared to etch the thin sections slightly. This pretreatment with aldehyde fuchsin caused no observable difference other than somewhat better contrast in the specimens studied. There was no change in the appearance of normal enamel in X-ray pictures, for instance. The increased contrast was helpful in interpreting detail in the very early zones of some lesions which were otherwise too faint to make out with confidence in all cases.

Microradiographs were made with a Phillips contact microradiographic unit (CMR-5). Exposures were made on Kodak 649-0 spectroscopic film, and developed for 5

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* Castolite, Castolite Company, Woodstock, Ill.

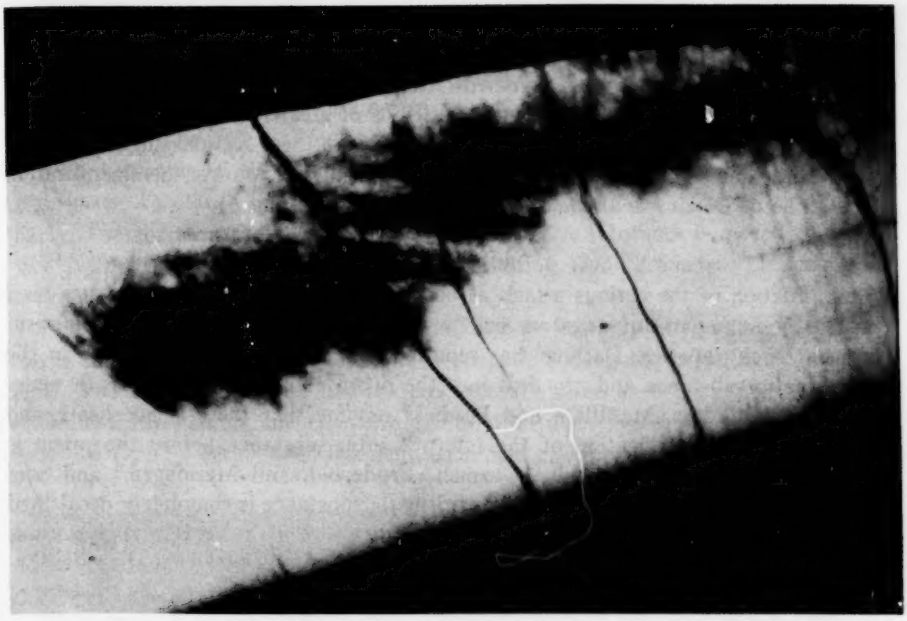

FIG. 2.—Microradiograph of same lesion as shown in Fig. 1. Note absence of dark positive zone seen in polarized light. The carious area shows considerable radiolucency in its central region, while peripheral areas appear much less demineralized. The demineralization can be seen to have progressed parallel to the long axes of the prisms. 4.5 kv., 2.0 mA. 145 ×.

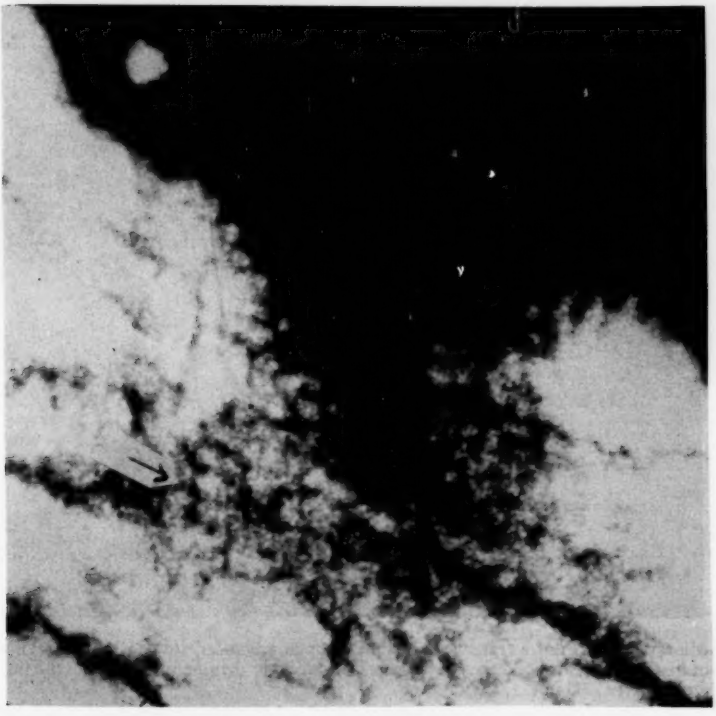

FIG. 3.—Microradiograph of an early zone at the deep edge of a lesion in transverse section. Note the round and arcade shapes of prisms outlined by demineralization at the prism periphery. 4.5 kv., 2.0 mA. 1,000×.

lucent rod substance is separated by narrow areas of much less mineral loss. The cross-striations are seen in longitudinal section as bands of greater density than the rest of the prism body. The thickness of the cross-striations in these sections suggests spread of the decalcification from these areas through the prism. Marked demineralization in the striae of Retzius is also seen at this stage (Figs. 2 and 5).

DISCUSSION

The initiation of the carious attack as occurring outside the prism body has been reported by numerous investigators on the basis of electron-microscope, polarized-light, and X-ray studies. Darling[2] has reported that demineralization begins in the interprismatic substance and proceeds into the prism core, leaving the sheath undemineralized until last. McMillan and Fosdick[6] assume that there is no sheath and report complete decalcification of the interprismatic substance before the prism is attacked. Bergman and Engfeldt,[1] Guzman, Brudevold, and Mermagen,[3] and Soni and Brudevold[4] also suggest that the interprismatic substance is completely decalcified before the prism bodies. The results of this study confirm the findings that carious demineralization begins outside the prism body, but they indicate that the interprismatic substance is the last structure to be completely demineralized. The results of

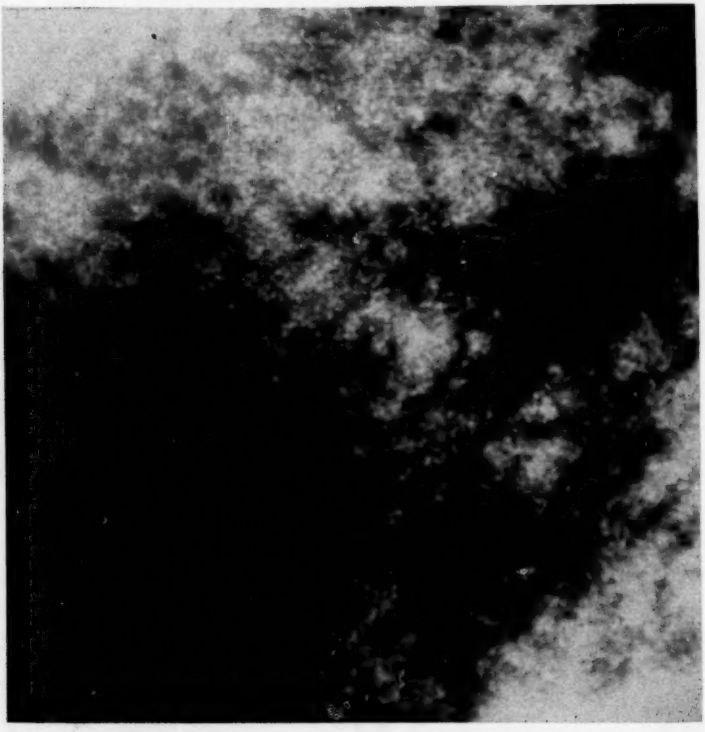

FIG. 4.—Microradiograph of a transverse section through advanced zone in central region of carious lesion shown in Fig. 3. Prism bodies are seen to be considerably demineralized, while the interprismatic substance is not. 4.5 kv., 2.0 mA. 1,000×.

the preceding report demonstrate that this early demineralization outside the rod body lies between the rod and the interprismatic substance in an area designated the "sheath space." The early carious demineralization in the "sheath space" appears to involve considerable of the interprismatic substance, as seen in Figure 3. Since the interprismatic substance never impregnates until after the rods, however, this seems unlikely. The apparent radiolucency in the interprismatic substance is more likely due to the poor resolution in microradiographs resulting from geometric blurring and photographic grain.

Thus the carious decalcification is initiated at the periphery of the prism, where it involves channels which permit its rapid propagation along the prism deep into the enamel. The striae of Retzius and the cross-striations are opened up next, carrying the decalcification across the enamel and into the prism bodies. Once exposed, the prisms are rapidly decalcified. Decalcification has also begun in the interprismatic

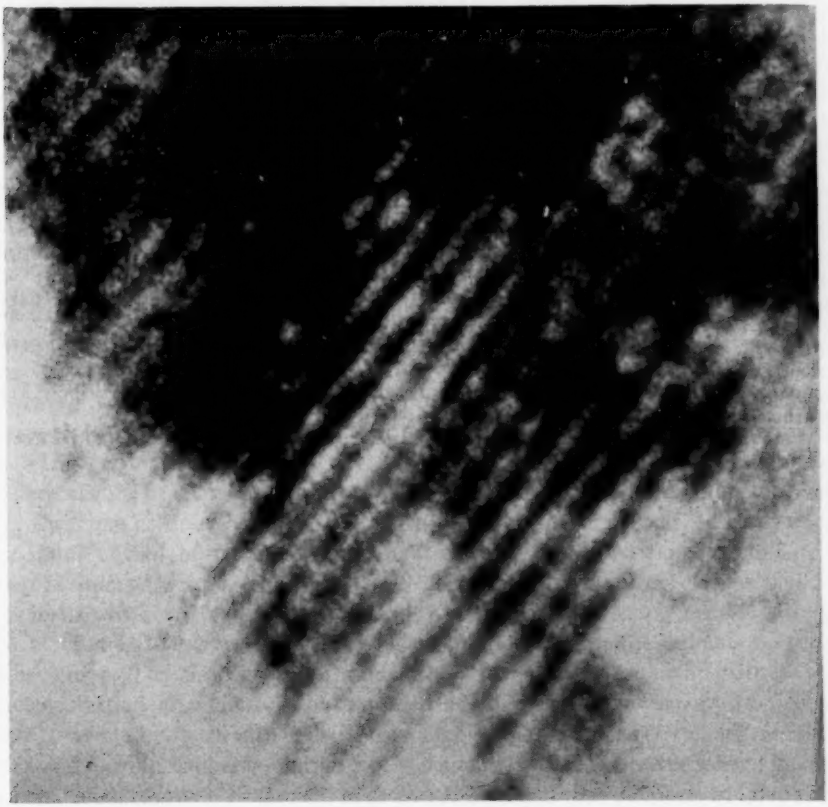

FIG. 5.—Microradiograph of longitudinal section through a carious lesion. The prisms are seen as highly demineralized areas, while the interprismatic substance is largely undemineralized. Cross-striations appear in the prism bodies as areas of greater demineralization than the remaining rod substance. The dark areas running vertically in the photographs are striae of Retzius. 4.5 kv., 2.0 mA. 1,000×.

substance by this time but proceeds much more slowly than in any of the other enamel structures.

The failure to obtain radiographs of the positive zone often described in polarized-light studies of caries and the translucent zones deep to this has been interpreted to mean that the demineralization of these areas is very slight. Darling[2] has reported similar findings with respect to these two zones, but Soni and Brudevold[4] have reported considerable demineralization in the positive zone. Although there seems to be little question that the translucent zone is an early carious area, further studies are needed to aid in understanding of the positive zone.

SUMMARY

Microradiographic studies with a Phillips contact microradiographic unit (CMR-5) were made of both early and advanced zones of white-spot smooth-surface lesions in longitudinal and transverse section.

These studies show that carious demineralization begins at the periphery of the rod, then involves the cross-striations, striae of Retzius, and prism body, leaving the interprismatic substance largely undemineralized until last.

We wish to thank Mr. Robert L. Smick, of the Phillips Electronic Corporation, for his kind cooperation.

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Effects of Vitamin A Deficiency on Experimental Carcinogenesis in Submandibular Glands of Hamsters

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A single injection of 7:12 dimethylbenz(α)anthracene dissolved in liquid petrolatum in various concentrations would cause production of sarcomas in the submandibular salivary glands of hamsters in 10–14 weeks.[1] Rowe and Gorlin[2] in their previous investigation showed that vitamin A deficiency promoted epithelial tumor production in the carcinogen-treated cheek pouch of the hamster. The present study was undertaken to investigate the effect, if any, of vitamin A deprivation on the incidence of tumors induced in the submandibular glands of the hamster.

MATERIALS AND METHODS

Eighty golden Syrian male hamsters (*Cricetus auratus*), 8 weeks of age, were divided into three groups of 50, 15, and 15 animals each. They were singly caged in a room maintained at a temperature of 72° ± 5° F. and a lighting regimen from 6:00 A.M. to 6:00 P.M. and darkness from 6:00 P.M. to 6:00 A.M. Under intraperitoneal Nembutal anesthesia, the mandibular glands were exposed in the fashion described by Chaudhry, Reynolds, Gorlin, and Vickers.[1] The right gland was injected with 0.05 cc. of 0.5 per cent 7:12 dimethylbenz(α)anthracene dissolved in liquid petrolatum; the left gland was used as control and was injected with the same amount of the solvent. The animals were fed Purina fox chow ad libitum prior to the injection, as well as postoperatively for 3 days, after which they were put on a vitamin A–deficient diet. The diet was prepared to order in the form of bread, and its contents were the same as described by Rowe and Gorlin.[2] In order to make certain that the bread was devoid of vitamin A, it was chemically analyzed repeatedly. The first group of animals was fed on vitamin A–deficient bread ad libitum. The second group of animals was also given the same bread, but the diet was supplemented with 1,000 I.U. of vitamin A per week. This group was pair-fed with the animals of the preceding group. The third group of animals was given the same bread with the same supplementation of vitamin A but was fed ad libitum. At the end of 18 weeks the animals were sacrificed with lethal doses of Nembutal, and their glands were extirpated and fixed in Zenker's solution for 24 hours. The tissues were processed in routine fashion, sections were examined microscopically, and the number of glands with tumors in each group was recorded. At the time of sacrifice, blood was drawn from

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each animal by means of cardiopuncture for serum vitamin A determination. Blood from 3 to 5 animals was pooled, and at least two different samples from each group were analyzed by the method described by Carr and Price.[3] Similarly, livers from 3 animals selected at random from each group were also removed for vitamin A level determination.

RESULTS

The number of animals which developed tumors in each group is shown in Figure 1. There was no difference in tumor yield between Groups II and III. Therefore, they were combined for the purpose of statistical analysis. The incidence of tumors in Group I was 25.5 per cent against 16.0 per cent in Groups II and III. This difference was statistically not significant, the probability being greater than 32.0 per cent. The average food consumption per animal per day was almost the same in all three groups of animals.

GROUP	NUMBER OF ANIMALS	NUMBER OF ANIMALS SURVIVED	NUMBER OF ANIMALS WITH TUMORS	% OF ANIMALS WITH TUMORS
I	50	35	9	25.5
II	15	13	2	15.4
III	15	12	2	16.6

FIG. 1.—Shows incidence of tumors in different groups of hamsters. Note that there is no significant intergroup difference in the incidence.

There was no intergroup difference in serum vitamin A and carotene levels, as is evident from Figure 2. On the other hand, vitamin A storage in the livers of Group I animals was significantly lower than in Groups II and III, the probability being 0.012. Again, there was no intergroup difference in the liver carotene, as shown in Figure 3.

DISCUSSION

Salley and Bryson[4] have demonstrated marked tissue alterations in salivary glands, both major and minor, in hamsters fed on vitamin A–deficient diet. The most characteristic change observed by them was metaplasia of the glandular epithelium into squamous variety. Similar metaplastic changes, as well as malignant epithelial tumors, were observed by Steiner,[5] Bauer and Byrne,[6] and Standish[7] following implantation of carcinogen in the form of pellets in the salivary glands of different animals. It was presumed that vitamin A deficiency and carcinogen combined might reinforce each

GROUP	SAMPLES	CAROTENE γ gm %	VITAMIN A γ gm %
I	1	< 20	18
	2	< 20	26
II	1	< 20	20
	2	< 20	33
III	1	< 20	20
	2	< 20	?

FIG. 2.—Indicates serum carotene and serum vitamin A level in different groups of hamsters

GROUPS	SAMPLES	CAROTENE γ gm/gm liver	VITAMIN A γ gm/gm liver
I	1	2.09	32.2
	2	2.66	16.9
	3	3.32	34.1
II	1	3.24	138.6
	2	2.46	383.3
	3	3.41	436.6
III	1	1.78	208.7
	2	3.15	386.6
	3	3.70	385.9

FIG. 3.—Shows carotene and vitamin A contents of liver in different groups of hamsters. Note depletion of vitamin A in animals of groups fed on vitamin A–deficient diet.

other's action, causing a higher tumor yield. The present investigation has failed to show any such evidence. This could possibly be due to the fact that the carcinogen when injected caused chemical destruction of the epithelial components beyond regeneration. The destroyed parenchymatous tissue was replaced by fibrous connective tissue, which, by the subsequent action of the carcinogen, resulted in the production of sarcomas. Since implantation of the carcinogen has resulted in the production of malignant epithelial tumor, it is conceivable that vitamin A deficiency may alter the tissue response when the latter mode of administration of the carcinogen is employed.

SUMMARY

Vitamin A deficiency does not enhance induction of malignant tumors in the submandibular salivary glands of the hamster when injected with a chemical carcinogen. This may be due to the fact that vitamin A deprivation primarily affects the epithelial components of the glands. When injected, the chemical toxicity of the carcinogen destroys the epithelial component, which becomes replaced by scar tissue, reactive fibrosis, and sarcoma. If the epithelial components are subjected to slow and gradual action of the carcinogen, the effects of vitamin A deficiency on the induction of tumor may become apparent. This may be achieved if the carcinogen is implanted in the form of pellets. It would, therefore, seem desirable to investigate the role of vitamin A deficiency when the latter mode of administration is employed.

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Studies in the Design and Analysis of Dental Experiments. III. Sequential Analysis (Double Dichotomy)

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In the usual type of experimental study, particularly those of a clinical nature, the number of subjects to be included is often determined by the availability of these subjects, of funds, or of the substance being studied. More "exactly," however, the size of the groups used should depend on the anticipated difference in treatment effects, how large (or small) a treatment difference is considered important to the investigator, and what magnitude of probability of error in rejecting or accepting the working null hypothesis (errors of the first and second kind) he is willing to tolerate. Methods for determining the sample size of experiments under various conditions have appeared in previous papers.[1, 2]

In many experiments, however, the investigator would like to have an idea of how the results are going before the preassigned total of subjects has been studied. Thus he may check the results and even perform significance tests partway through the study. Some decision will be made as to discontinuance of the study because it does not look promising or because a difference is already apparent, or else to continue the study because it looks promising. Unfortunately, however, he often does not realize that by this procedure he is changing his agreed-upon probabilities of errors of the first and second kind. A desire to take stock of the situation periodically and still control the probabilities of error leads logically to a sequential type of design. Finney has stated:

> The use of a time sequence of pairs suggests a sequential design. If the results for any subject are obtainable fairly rapidly, any large difference in effectiveness of A and B is likely to betray itself from tests on only a few pairs: to continue until a preassigned number has been tested not only seems uneconomic experimentation but also offends against the ethical principle that a remedy shall not be used after it has been proved inferior. On the other hand, if the difference between A and B is small, a preassigned number of subjects may fail to point decisively to either as the better, and to stop the experiment at that total could be almost equivalent to wasting all the work already done. In practice, most clinical experimenters no doubt decide whether to continue or to end an experiment from study of the results already obtained, and what is wanted is an objective rule of conduct.[3]

During the second World War the need for determining as rapidly as possible which of two products was better gave impetus to the development of the theory and application of sequential analysis by Wald and his co-workers.[4, 5] Since that time, a number of

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papers dealing with the applications of these methods in medical experimentation have appeared in the literature.[6–15] It is the purpose of this paper to explore the application of these statistical techniques to experiments in the dental field.

DESCRIPTION OF THE EXPERIMENT

An experiment was designed to determine the relative effectiveness of 5 per cent lidocaine in two different ointment bases as a topical anesthetic. Previous experience had indicated that 5 per cent lidocaine in a mineral oil–polyethylene base* ointment would produce discernible topical anesthesia of oral mucous membranes about 70 per cent of the time. The investigators thought that an increase of 15 per cent (from 70 to 85 per cent) in the performance of the lidocaine in gelatin pectin, carboxymethyl cellulose in mineral oil–polyethylene base ointment,† compared to lidocaine in Plastibase, would be of practical importance. With these base-line data, a sequential scheme can be developed.

TABLE 1

ORDER OF APPLICATION OF TWO TOPICAL ANESTHETIC PREPARATIONS TO PAIR MEMBERS

Pair No.	Preparation Used		Pair No.	Preparation Used	
	1st Member	2d Member		1st Member	2d Member
1	A	B	11	B	A
2	B	A	12	A	B
3	B	A	13	A	B
4	B	A	14	A	B
5	A	B	15	A	B
6	A	B	16	A	B
7	B	A	17	A	B
8	B	A	18	A	B
9	A	B	19	B	A
10	A	B	20	A	B

The two preparations were applied to the oral mucous membranes of patients under treatment at the School of Dental and Oral Surgery, Columbia University, in a random fashion, so that half the subjects had preparation A (Orabase vehicle) on the right side of the mouth and the other half preparation B (Plastibase vehicle). For each pair of subjects a decision was made (by a coin toss) as to which one received A on the right side and which one B. The sequence of drug application appears in Table 1. The preparations were applied in a double-blind manner—neither the subject nor the investigators knew the composition of the preparations applied.

The effectiveness of topical anesthesia was determined at various time intervals, but only the observations at 12 minutes after application are presented here, because this was chosen at the beginning of the experiment as the critical comparison. The presence or absence of anesthesia was determined for each patient, and then a comparison was made between the two members of each pair. Thus there are four possibilities for the

* Plastibase.

† Orabase.

outcome of a pair: (1) A produced anesthesia while B did not; (2) B produced anesthesia while A did not; (3) neither A nor B produced anesthesia; (4) both A and B produced anesthesia. Alternatives 3 and 4 yield no information as to the relative effectiveness of the preparations and are referred to as "tied pairs." Only alternatives 1 and 2, the "untied pairs," give information about the relative value of the preparations, i.e., A is better than B or A is not better than B (actually A is worse than B). The tally of the results appears in Table 2.

TABLE 2

THE RELATIVE EFFECTIVENESS OF TWO TOPICAL ANESTHETIC PREPARATIONS (A AND B) FOR EACH PAIR OF PATIENTS, RIGHT SIDE, 12 MINUTES AFTER APPLICATION

Pair No.	A Effective B Ineffective	B Effective A Ineffective	A and B Both Ineffective	A and B Both Effective
1		×		
2	×			
3	×			
4	×			
5	×			
6				×
7				×
8	×			
9			×	
10	×			
11	×			
12		×		
13				×
14	×			
15	×			
16	×			
17	×			
18			×	
19				×
20	×			

THE SEQUENTIAL PLAN

In preparing the sequential plan for this experiment, the following considerations entered: (1) past experience indicated that 5 per cent lidocaine in a Plastibase vehicle (preparation B) produced topical anesthesia of the oral mucous membranes in about 70 per cent of the cases, i.e., $p_1 = 0.70$; (2) the investigators felt that an increase in the percentage of patients having topical anesthesia with the new preparation A of 15 per cent was important, i.e., $p_2 = 0.70 + 0.15 = 0.85$; (3) the investigators then chose appropriate probabilities of errors of the first kind (α) and of the second kind (β). They took $\alpha = 0.05$, i.e., they were willing to take a chance of 5 in 100 of saying that the true difference ($p_2 - p_1$) was greater than zero when it was, in fact, zero (or even less than zero, in which case A was actually less effective than B). More broadly, they were willing to take this chance of declaring significance, when, in fact, the true difference was zero (or less than zero).

They chose $\beta = 0.20$, i.e., they were willing to take a chance of 20 in 100 of saying

that the true difference was not greater than zero, when, in fact, it was 15 per cent. More broadly, they were willing to take this chance of declaring non-significance when the true difference was 15 per cent as specified. The test is said to have a power of 0.80 ($= 1 - \beta$) or 80 per cent. That is, if the p_1 and p_2 are as stipulated, the probability of finding significance in the experiment is 80 per cent.

Utilizing the above criteria, the sequential plan was calculated as follows:

Let $\theta =$ proportion of untied pairs favorable to preparation A. Then

$$\theta = \frac{p_2 q_1}{(p_2 q_1 + p_1 q_2)}$$

where

$$p_1 = 0.70 \quad \text{and} \quad q_1 = 1 - 0.70 = 0.30 ,$$

$$p_2 = 0.85 \quad \text{and} \quad q_2 = 1 - 0.85 = 0.15 .$$

Thus

$$\theta = \frac{(0.85)(0.30)}{[(0.85)(0.30) + (0.70)(0.15)]} = 0.7083 .$$

Actually, the same θ corresponds to other combinations of p_1 and p_2 differing by more than 15 per cent (for p_1 less than 70 per cent) and by less than 15 per cent (for p_1 greater than 70 per cent). If $p_2 = p_1$, then, of course, $\theta = \frac{1}{2}$. (If A is actually less effective than B, so that $p_2 < p_1$, then, of course, $\theta < \frac{1}{2}$.)

We also calculate the following:

$$a = \log \frac{(1-\beta)}{\alpha} = \log \frac{0.80}{0.05} = \log 16 = 1.20412 ,$$

$$b = \log \frac{(1-\alpha)}{\beta} = \log \frac{0.95}{0.20} = \log 4.75 = 0.67669 ,$$

$$c = \log \frac{1}{2(1-\theta)} = \log \frac{1}{2(0.2917)} = \log 1.7141 = 0.23403 ,$$

$$d = \log \frac{\theta}{(1-\theta)} = \log \frac{(0.7083)}{(0.2917)} = \log 2.4282 = 0.38529 .$$

We calculate the boundary lines for the sequential plan which must be crossed before a decision can be reached:

Reject $\theta = \frac{1}{2}$ and declare preparation A is better than B when $y > a/d + (c/d)n$, where y is the number of untied pairs favoring the new preparation A and n is the number of untied pairs.

Reject $\theta = 0.7083$ and declare preparation A not better than B by 15 per cent when $y < -b/d + (c/d)n$. Thus the lines are calculated to be as follows:

$$y = 3.125 + 0.6074n \quad \text{and} \quad y = -1.756 + 0.6074n .$$

These two lines are drawn on coarse crosshatched arithmetic graph paper (Fig. 1). On the abcissa scale, n is plotted as the number of untied pairs, and on the ordinate scale y is plotted as the number of these pairs favorable to the new preparation, A.

From the results in Table 2, we note that the first pair is untied, favoring preparation B. On the sequential graph, we plot one unit to the right on the abcissa for an untied pair. The next pair is also untied but favoring preparation A, so we go one unit to the right on the abcissa scale for an untied pair and one unit up on the ordinate scale for an untied pair favoring preparation A. The next three pairs are also untied, favoring preparation A, so in each case we go one unit to the right and one up. The next two pairs are tied, in that both preparations produced satisfactory anesthesia, and are not recorded on the graph. The eighth pair is untied, favoring A, so again we go one unit right and one up, while the ninth, being tied, with both preparations being ineffective, is not recorded. The tenth and eleventh pairs are untied, favoring A, and are each

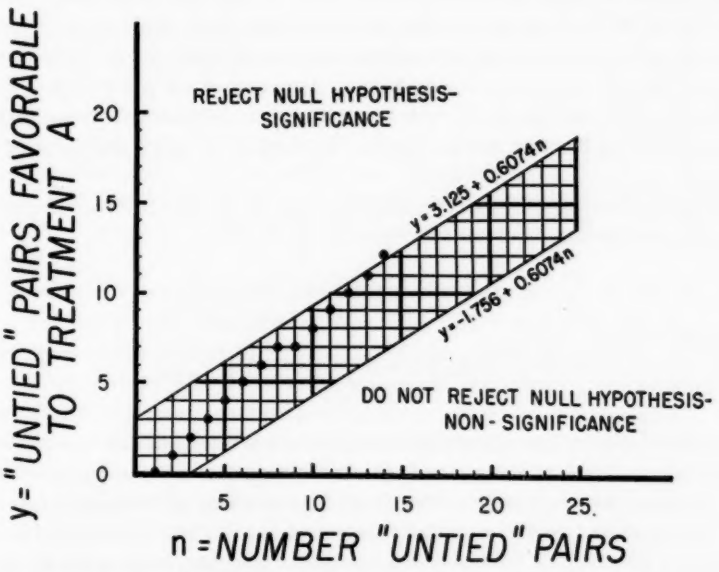

FIG. 1.—One-sided sequential plan, $\alpha = 0.05$; $\beta = 0.20$; $\theta = 0.7083$

recorded one unit to the right and one up. The twelfth pair is untied, favoring B, and is recorded one unit to the right. The thirteenth pair is tied and is not recorded. We continue in the above manner until we record the twentieth pair, which is untied, favoring A, at which point the upper line is crossed. This then terminates the experiment. The decision is to reject the null hypothesis, declaring that preparation A is better than preparation B under the conditions set forth at the beginning of the experiment. In other words, the observed proportion of the A cases showing effective topical anesthesia is significantly higher than the corresponding proportion of the B cases.

If the tracks crossed the bottom line, it would be concluded that preparation A was not better than preparation B (or, rather, not better by 15 per cent). The tracks may wander for some time between the upper and lower lines, but they will eventually cross one of them, no matter what the true difference between the preparations may be.

ESTIMATION OF SAMPLE SIZE

It is possible to estimate the average sample size (ASN) necessary to arrive at a decision, i.e., to cross one of the two lines in sequential analysis. When there is no difference between the treatments, so that $\theta = \frac{1}{2}$, we calculate from the formula

$$n = \left[\frac{a}{d} - \alpha\left(\frac{a}{d} + \frac{b}{d}\right)\right] \div \left(\frac{c}{d} - \frac{1}{2}\right).$$

Substituting,

$$n = \frac{1.765 - 0.05\,(4.882)}{0.1074} = 14\,,$$

where n is the number of untied pairs. Thus, if the null hypothesis is true, that $p_1 = p_2$, then, on the average, we will need 14 untied pairs to arrive at a decision. For 95 per cent of the time, this decision will be one of non-significance. If the ratio, $\theta = \frac{1}{2}$, is produced by $p_1 = p_2 = 0.70$, so that 42 per cent of all pairs $[(p_2q_1 + p_1q_2) = (0.7 \times 0.3 + 0.7 \times 0.3) = 0.42]$ will be untied, this indicates that the total number of pairs needed, on the average, is equal to $14/0.42 = 33$ pairs, and hence 66 individuals.

If the alternative hypothesis corresponding to $\theta = 0.7083$ is true, we can calculate the average sample size from the formula

$$n = \left[\frac{b}{d} - \beta\left(\frac{a}{d} + \frac{b}{d}\right)\right] \div \left(\theta - \frac{c}{d}\right).$$

Substituting,

$$n = \frac{3.1252 - 0.2\,(4.8815)}{0.1009} = 21\,.$$

If the specified alternative hypothesis is true, we will need, on the average, 21 untied pairs to reach a decision. Since $\beta = 0.20$, this means that 80 per cent of the time the decision will be that A is better than B. If this specified alternative hypothesis that $\theta = 0.7083$ is produced by $p_1 = 0.70$ and $p_2 = 0.85$, then $[(0.70)(0.15) + (0.85)(0.30)] = 36$ per cent of the pairs will be untied, and hence the total number, N, of pairs is $21/0.36 = 58$, on the average. This requires a total of 116 individuals.

It should be pointed out that if the true situation is somewhere in between the null hypothesis ($\theta = \frac{1}{2}$) and the postulated alternative hypothesis ($\theta = 0.7083$), the average amount of testing will be maximized. The average sample size would be somewhat greater than if either the null or the alternate hypothesis were true.

If, instead of employing sequential analysis, the method of fixed sample size were used, the estimated sample sizes could be calculated by using methods described previously for the unpaired situation.[1, 2] Using a one-tailed test, $\alpha = 0.05$, $\beta = 0.20$, and $p_1 - p_2 = 15$ per cent, we calculate

$$\sqrt{N} = \tfrac{1}{15}\{1.64\sqrt{[2\,(77.5\%)\,(22.5\%)]} + 0.84\sqrt{[(70\%)\,(30\%) + (85\%)\,(15\%)]}\}\,,$$

where N is approximately 94 individuals in each group, or a total of 188 individuals.

SEQUENTIAL SAMPLING VERSUS FIXED SAMPLES

Advantages of sequential sampling.—There are two basic advantages of the sequential method. First, as has been shown above, the average sample size necessary to

assert significance is less than the necessary sample size calculated on the basis of a fixed sample size. Second, if the difference between the two preparations, A and B, exceeds that specified in the preparation of the plan or if, in fact, A is worse than B, so that p_2 is less than p_1, a decision is usually reached much sooner.

Disadvantages of sequential sampling.—One of the basic diasadvantages is that the nature of the study may not lend itself readily to this method, as, for example, when there is an appreciable time lag between the (intake) treatment of the subjects and the evaluation of the results, so that many subjects may still be in the process of evaluation when a decision is reached. Another problem sometimes encountered is that, because of random variation, the testing may go on much longer than the calculated average sample size. To circumvent this difficulty, the investigator can decide in advance to stop the experiment (truncation) at, for example, twice the calculated fixed sample size and make the decision corresponding to the nearest line. If this truncation is performed far out on the sequential graph, such as at twice the fixed sample size, it does not materially change the values previously selected for α and β.[5]

One further disadvantage of the sequential method is largely an administrative one. In setting up a study it is often necessary to prepare a suitable budget and also to hire additional personnel, such as technicians. These people are usually hired for a definite period of time. If the study is terminated more rapidly than originally planned or if it continues longer than originally planned, the problem arises as to what to do with the staff.

Conventional significance test on sequential data.—One might inquire whether significance could be asserted if a conventional significance test were performed on this sample which was large enough to arrive at a decision of significance by the sequential procedure. If we were to take these data and pretend that they corresponded to a predetermined sample size, performing an ordinary significance test (which is, of course, not legitimate), we would expect to obtain statistical significance except, perhaps, in the case of unusual values of α and β. The conventional test[16] uses the untied pairs and compares the proportion in which preparation A is effective and preparation B is ineffective with ½. Twelve of the 14 untied pairs in Table 2 favor preparation A and 2 favor preparation B. Computing χ^2 with a continuity correction,[17] we obtain

$$\chi^2 = \frac{[(12-2)-1]^2}{12+2} = \frac{81}{14} = 5.79 .$$

The critical value of χ^2 corresponding to the 5 per cent level of probability for a one-tailed test is 2.71. Consequently, we have statistical significance well beyond the 5 per cent level.

The fact that the conventional test exhibits greater statistical significance than that obtained by the sequential test does not represent a disadvantage of the sequential procedure. After all, how would one have known exactly where to stop the experiment to perform this test? If the investigator were to stop repeatedly to peek at the data and make this conventional test, he would be losing control over the errors of the first and second kind.

A TWO-SIDED SEQUENTIAL PLAN

In the previous sequential plan for comparing the effectiveness of two topical anesthetic preparations, we were interested only in determining whether preparation A was

better than preparation B and not whether B was better than A. This one-sided test is frequently encountered in clinical research when a new treatment is to be compared with an established treatment. Sometimes, however, the investigator has two competing treatments or preparations, and he wants to know which one is the better. This latter situation requires the setting-up of a two-sided plan.

The simplest two-sided sequential plan is to combine two one-sided sequential plans: Plan 1 to test whether A is *better* than B as previously illustrated, and Plan 2 to test whether A is *worse* than B (this latter is equivalent to testing whether B is better than A). If the investigator selects the same criteria as for the one-sided plan illustrated ($\alpha = 0.05$, $\beta = 0.20$, and $\theta = 0.7083$), then it will be necessary to apportion half of the α to each side of the plan, so that the new α for each of the one-sided plans to be constructed will be 0.025.

Plan 1 will provide the two lines for the upper portion of the two-sided plan. In this plan, $\alpha = 0.025$, $\beta = 0.20$, and $\theta = 0.7083$. We calculate

$$a = \log \frac{(1-\beta)}{\alpha} = \log \frac{0.80}{0.025} = \log 32 = 1.50515,$$

$$b = \log \frac{(1-\alpha)}{\beta} = \log \frac{0.975}{0.20} = \log 4.875 = 0.68797,$$

$$c = \log \frac{1}{2(1-\theta)} = \log \frac{1}{2(0.2917)} = \log 1.7141 = 0.23403,$$

$$d = \log \frac{\theta}{(1-\theta)} = \log \frac{0.7083}{0.2917} = \log 2.4282 = 0.38529.$$

The lines for the upper portion of the two-sided sequential plan are calculated as follows:

$$y = \frac{a}{o} + \frac{c}{d} n = 3.907 + 0.6074n, \quad \text{and} \quad y = -\frac{b}{d} + \frac{c}{d} n = -1.786 + 0.6074n,$$

where y is the number of untied pairs favoring preparation A and n is the number of untied pairs.

Plan 2 will provide the two lines for the lower portion of the two-sided plan. Using $\alpha = 0.025$, $\beta = 0.20$, and $\theta = 0.7083$, we obtain the same values for a, b, c, and d as for Plan 1. The lines for the lower portion of the two-sided plan, then are

$$z = \frac{a}{o} + \frac{c}{a} n = 3.907 + 0.6074n, \quad \text{and} \quad z = -\frac{b}{d} + \frac{c}{d} n = -1.786 + 0.6074n,$$

where z is the number of untied pairs favoring preparation B and n is the number of untied pairs. Since y, the number of untied pairs favoring preparation A, is n–z, the equations of the two lower lines in terms of y, which are plotted in Figure 2, are

$$y = n - z = n - (3.907 + 0.6074n) = -3.907 + 0.3926n,$$

$$y = n - z = n - (-1.786 + 0.6074n) = 1.786 + 0.3926n.$$

The use of the same α, β, and θ for Plans 1 and 2 results in a rather convenient type of symmetry in the equations of the four lines determining the sequential plan (Fig. 2).*

The four lines resulting from Plans 1 and 2 are drawn on coarse crosshatched arithmetic graph paper, shown in Figure 2. The data from Table 2 are recorded on the graph in the manner described previously. It will be noted, however, that the fourteenth (last) untied pair does not cross the upper line of the graph as it did in the one-sided sequential plan depicted in Figure 1. Consequently, we cannot say that preparation A is significantly different from preparation B at the 5 per cent level of significance. It is evidentally necessary to continue the experiment to obtain a greater number of untied pairs, until one of the lines is crossed, in order to be able to make a decision.

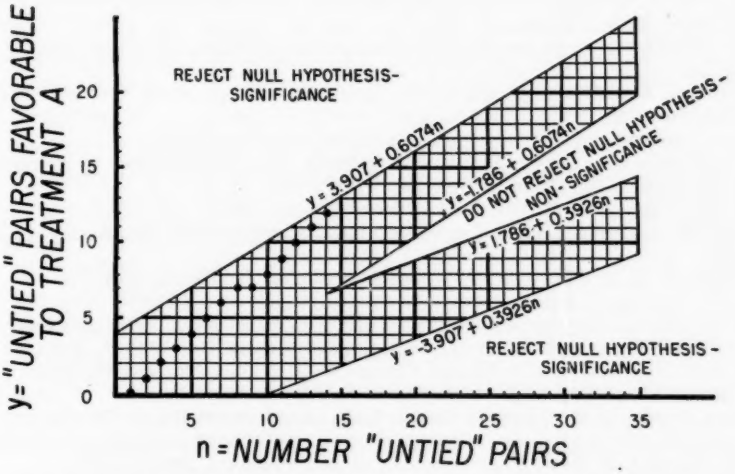

FIG. 2.—Two-sided sequential plan, $\alpha = 0.025$; $\beta = 0.20$; $\theta = 0.7083$

If the upper line were crossed, the null hypothesis of no difference would be rejected, i.e., significance would be declared, and it would be stated that preparation A is better than preparation B. If the lower line were crossed, the null hypothesis would be rejected, and the statement would be made that A is worse than B (B is better than A). On the other hand, if the path of points moved into the central triangular zone, the null hypothesis would not be rejected, meaning that the difference between A and B, positive or negative, is less than 15 or 21 per cent, respectively (i.e., when $p_1 = 70$ per cent), and is not statistically significant.

* The use of the same θ for Plans 1 and 2 does not, however, result in the same difference between p_1 and p_2 for each of these plans. Thus, for Plan 1, $p_2 - p_1 = 85$ per cent $- 70$ per cent $= 15$ per cent, while for Plan 2, $p_2 - p_1 = 49$ per cent $- 70$ per cent $= -21$ per cent. This latter difference is related to the value of θ used by the formula

$$p_2 - p_1 = \frac{(p_1 q_1)(1 - 2\theta)}{\theta q_1 + (1 - \theta) p_1}.$$

SUMMARY

The application of sequential analysis to an experiment on the relative effectiveness of two preparations of topical anesthetics in the oral cavity has been described. The advantages and disadvantages of this type of analysis as compared with that usually employed in clinical experimentation have been discussed. The relative merits of a one-sided versus a two-sided significance test were discussed and illustrated for the sequential design.

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A Study of the Exfoliative Cytology of the Hard Palate and Buccal Mucosa Following Cessation of Smoking in Previous Smokers

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For many years investigators have tried to isolate the irritating properties found in cigarette smoke. Flinn[1] wrote in 1935 that he had conducted experiments with 100 individuals, testing two hygroscopic agents found in cigarettes. The results showed that glycerine was more irritating than diethylene glycol. Flinn's studies were an elaboration of the work done by Mulinos and Osborne.[2] They had tested the irritating properties of the above-mentioned hygroscopic agents on the conjunctiva of rabbit eyes. It was noted then that glycerine was much more irritating than diethylene glycol. Ballinger and Johnson[3] question the validity of their findings. Kreshover and Salley,[4] in studying the effects of cigarette smoke on the oral tissue of mice and hamsters, noted no significant changes in these structures in their experimental procedures. Pindborg,[5] studying human subjects, found some correlation between the use of tobacco and inflammatory changes in the gingiva.

During the last 10 years, studies of the keratinization of the oral mucosa have been conducted by means of exfoliative cytology techniques. Montgomery[6] showed that age, sex, and menstrual cycle did not significantly influence the "cell pattern" of the oral mucosa. Miller, Soberman, and Stahl[7] demonstrated that the palate was the most highly cornified tissue of the oral cavity, the gingiva the next most highly cornified tissue, the dorsal of the tongue next, and the cheek least of all. This pattern remained constant through various ages.

The present study will, by means of exfoliative cytology, investigate the keratinization of the hard palate and cheek following cessation of smoking in previous smokers. It will be further noted whether the cell pattern of the hard palate and cheek varies significantly from one individual to another.

MATERIALS AND METHODS

Eleven male New York University Dental School students ranging in age from twenty-one to thirty-five years, who had smoked one to one and a half packs of cigarettes per day during the previous year, volunteered to discontinue smoking for a period of 4 weeks. Oral scrapings of the hard palate and the cheek of the smokers were taken on alternate days for a period of 2 weeks prior to cessation of smoking. These scrapings were immediately placed on marked slides and fixed in dehydrated alcohol. The subjects then stopped smoking for 4 weeks, and scrapings of the above-mentioned areas were obtained in a similar manner. At the end of 6 weeks, five indi-

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viduals did not continue with the experiment, since they did not want to resume smoking. The remaining volunteers resumed smoking, and scrapings were obtained as outlined above for 2 successive weeks.

All specimens were processed as described by Papanicolaou.[8] The material was then evaluated and classified as outlined by Miller, Stahl, and Soberman.[7]

The findings were based on a 100 cell count from each scraped area in each individual. Cells were divided into yellow-staining anucleated cells, yellow-staining nucleated cells, red-staining nucleated cells, and green-staining nucleated cells.

The average number of yellow-staining anucleated cells of the palate and green-staining cells of the buccal mucosa were computed in the same individual (*a*) while smoking, (*b*) when smoking ceased, and (*c*) when smoking was resumed.

Statistical changes of significance were computed by the following formula:

$$\text{Critical Ratio} = \frac{\text{difference of the average of one phase of the experiment to another}}{\text{standard error of the difference}}.$$

If the critical ratio was above 2, it was considered significant.

RESULTS

Palate.—In the palatal mucosa, eight individuals had relatively little variation in the averages of yellow-staining anucleated cells from one phase of the experiment to another. Three men showed statistically significant variation in the averages of yellow-staining anucleated cells when they ceased smoking. In one of these three individuals the anuclear yellow count increased, and in two individuals it decreased. One of the volunteers demonstrated wide variation in the average anuclear yellow cell count from the beginning to the end of the experiment (Table 1).

Buccal mucosa.—Seven individuals showed no significant increase or decrease in the average number of green-staining cells from the first phase of the experiment to the second phase. Two of the remaining four men had significant increases in the averages of green-staining cells, and two decreased significantly. Two volunteers showed significant increases in the averages of green-staining cells when comparing the first and third phases of the experiment. One of these men had shown significant changes when comparing the first two phases of the experiment. When comparing the second and third phases of the experiment, two showed significant decreases in the averages of the green-staining cells, and one significantly increased. One of the three also showed significant changes when comparing the first and third phases of the experiment (Table 2).

When the average percentage of yellow-staining anucleated cells of one individual's palate was compared with the averages of other men in the experiment, 11 readings out of a possible 55 showed significant differences (Table 3).

When similar comparisons of green-staining cells of the buccal mucosa were made, 12 readings out of 55 indicated significant differences. Therefore, no definite pattern could be reported in either comparison (Table 4).

DISCUSSION

In Miller, Soberman, and Stahl's[9] studies of oral exfoliative cytology it was noted that a definite cellular pattern existed for specific areas of the oral cavity. They further

TABLE 1

NUMBER OF PATIENTS WITH STATISTICALLY SIGNIFICANT CHANGES IN YELLOW-STAINING CELLS FROM PALATAL MUCOSA RELATED TO SMOKING HABITS

	M_1–M_2*	M_1–M_3	M_2–M_3
Increased	1	1	1
Decreased	2	0	0
Total no. of significant changes	3	1	1

* Smoking habits: M_1 = 2 weeks of smoking; M_2 = 4 weeks when smoking ceased; M_3 = 2 weeks when smoking resumed.

TABLE 2

NUMBER OF PATIENTS WITH STATISTICALLY SIGNIFICANT CHANGES IN GREEN-STAINING CELLS FROM BUCCAL MUCOSA RELATED TO SMOKING HABITS

	M_1–M_2*	M_1–M_3	M_2–M_3
Increased	2	0	1
Decreased	2	2	2
Total no. of significant changes	4	2	3

* Smoking habits: M_1 = 2 weeks of smoking; M_2 = 4 weeks when smoking ceased; M_3 = 2 weeks when smoking resumed.

TABLE 3

COMPARISONS OF AVERAGE NUMBER OF ANUCLEATED YELLOW-STAINING CELLS OF HARD PALATE BETWEEN INDIVIDUALS BY MEANS OF CRITICAL RATIOS

		A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}
Wrubel	A_1		1.3	1.7	2*	3.7*	3.5*	3*	1.1	0	0.8	2.1*
Ben	A_2			0.2	0.4	1.5	1	0.7	0.2	1.3	0.4	0.6
Bakke	A_3				0.3	1.7	1	0.6	0.4	1.7	0.7	0.1
Arbuse	A_4					1.6	0.6	0.1	0.7	2*	0.9	0.3
Gottesman	A_5						0.6	2.1*	1.6	3.4*	1.9	1.9
Drazner	A_6							0.9	1.1	2.9*	1.1	1.2
Taubkin	A_7								0.9	2.6*	0.8	0.6
Roemer	A_8									1	0.2	0.2
Pollack	A_9										0.7	2*
Fishbein	A_{10}											0.8
Sippel	A_{11}											

*Critical ratio above 2.

TABLE 4

COMPARISONS OF AVERAGE NUMBER OF GREEN-STAINING CELLS FROM BUCCAL MUCOSA BETWEEN INDIVIDUALS BY MEANS OF CRITICAL RATIOS

		A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}	A_{11}
Sippel	A_1		3.4*	1.7	1.9	2.4*	3.4*	3.0*	4.7*	3.1*	4.1*	3.4*
Wrubel	A_2			2.5*	1.7	1.8	1	0.9	0.3	1	0.2	0.2
Ben	A_3				2.1*	0.5	1.3	1.4	2.5*	1.3	2.5*	0.1
Bakke	A_4					0.1	1	0.9	1.7	0.8	1.8	1.6
Arbuse	A_5						0.9	0.9	1.9	0.8	1.9	1.7
Gottesman	A_6							0.1	0.9	0.1	1.4	0.9
Drazner	A_7								0.8	0.1	1.1	0.9
Taubkin	A_8									1.1	0.5	0.4
Pollack	A_9										1.2	1.1
Roemer	A_{10}											0.2
Fishbein	A_{11}											

* Critical ratio above 2.

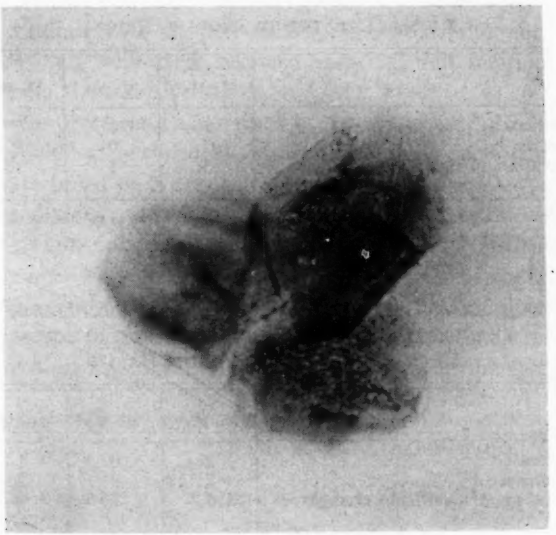

FIG. 1.—Photomicrograph of anucleated yellow-staining cells obtained from the hard palate. Papanicolau stain. Mag. 500×.

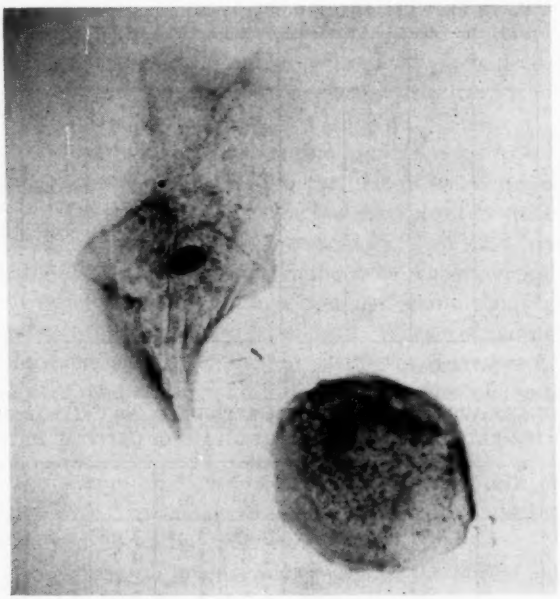

FIG. 2.—Photomicrograph of anucleated green-staining cells obtained from the buccal mucosa. Papanicolau stain. Mag. 500×.

reported that the constancy of the pattern was not altered by aging. Montgomery[6] also found a constancy in the cytologic patterns in various regions of the mouth that were statistically significant. The present findings confirm the existence of a keratinization pattern that appears to be specific for various areas of the oral mucosa.

A comment should be made regarding the inconsistency of the cytologic findings following cessation of smoking. While differences in the oral exfoliative pattern of some individuals were noted during the experiment, no clear trend could be established. This inconsistency may be due to (1) a lack of response of the oral epithelium to variations in smoking habits; (2) an inadequacy of the sample tested in both number of volunteers and duration of the experiment; or (3) an improper choice of the sampling site. Further studies are necessary in order to clarify these factors.

SUMMARY

Oral exfoliative cytology was employed, in order to observe changes in keratinization of the palate and buccal mucosa following cessation of smoking.

Eleven male New York University College of Dentistry students, varying in age from twenty-one to thirty-five years, volunteered for this study.

Scrapings were taken on alternate days of the palate and buccal mucosa for (1) 2 weeks while the men continued smoking; (2) 4 weeks while the men ceased smoking; and (3) 2 weeks when the men resumed smoking.

No definite change in the pattern of oral exfoliative cytology could be observed following the cessation of smoking in our group of volunteers.

When the average cell count of each individual was compared with the findings of the entire group, the average number of anucleated yellow-staining cells and green-staining cells did not present a specific pattern for each individual.

These findings do not preclude the possibility that smoking may affect oral keratinization patterns; however, under the limitations of this experiment, this difference could not be ascertained.

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The Endogenous Respiratory Quotient of Bovine Dental Pulp

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Although the respiratory quotient (R.Q. $= Q_{CO_2}/Q_{O_2}$) is inadequate for the specification of biochemical details, it does express in a general manner some of the more superficial qualities of respiratory metabolism. It has been used most especially to indicate the character of nutritional substances undergoing intracellular oxidation, since it is a numerical statement of the relation of the amount of oxygen consumed in converting the oxidizable material to a measured amount of excreted carbon dioxide. Complete oxidative metabolism of carbohydrate should, on theoretical grounds, produce an R.Q. of 1.0, and this has been closely substantiated by laboratory studies. Similar metabolism of protein should produce an R.Q. between 0.9 and 0.8. The R.Q. for fat metabolism should lie between 0.8 and 0.7. These values are approximate and vary with the methods and materials employed in determining, them.[1–4]

Our interest in the respiratory quotient of dental pulp was stimulated by studies previously reported from our laboratory[5] indicating the ability of this tissue to survive in vitro for many hours without exogenous nutritive support while it continued to consume oxygen at reasonably normal levels. The question raised by this behavior is whether the pulp tissue, isolated from an active circulation, continues to derive its energy from free or loosely bound carbohydrates of its connective tissue matrix or is forced to consume its lipid and protein constituents for this purpose. It was believed that a partial answer to this question might be found if a satisfactory method could be devised that permitted continuous measurements of oxygen consumption and carbon dioxide production over a period of several hours. An especially desirable feature of such a method would be the accomplishment of both types of measurement on the same tissue samples. This is desirable because of the frequently low activity of dental pulp that produces small manometer changes sometimes approaching the lower limit of reliability of the method. Under such conditions, slight variations in metabolic activity of tissue samples contained in different flasks, as required by both the direct and the indirect method of Warburg,[6] could produce erroneous respiratory quotients. It was recognized that all convenient methods presently available fail to distinguish between respiratory carbon dioxide and acid products of metabolism that displace carbon dioxide from the buffer system of the suspending fluid and that any respiratory quotient data would require further investigation to disclose their full significance.

Reports on the respiration of dental pulp by Pincus,[7] Kozam and Burnett,[8] and

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Sasaki[9] have indicated that the R.Q. of this tissue lies between 0.9 and 1.1. These investigators studied human, rat and rabbit, and dog pulp tissue, respectively. Their relatively consistent findings strongly suggest that carbohydrate utilization is prominent in the metabolism of this organ.

MATERIALS AND METHODS

The tissue used in this study was pulp from molar teeth of freshly killed cattle. The pulp was classified, as in our previous studies, as to the arbitrary developmental status of the tooth from which it was taken.[5] *Mature* teeth were those in which root formation was complete; *young* teeth were those in which root formation was in progress; and *embryonic* teeth were those in which root formation was not yet well established. Tissue slices used in this study were approximately 0.5 mm. thick and were made with a Stadie-Riggs tissue slicer.

The reaction vessels were Stanley-Tracewell respiratory quotient flasks[10] of approximately 6-ml. capacity. These flasks have two side wells for the CO_2 absorbent communicating with the main chamber of the vessel through large-aperture stop-cocks that permit control of CO_2 absorption at will. Preliminary studies with pulp tissue suggested that oxygen consumption was modified by the presence of CO_2 in the gas phase. Consequently, CO_2 absorption during Q_{O_2} determinations was accomplished by the use of the reversible "CO_2 buffer" containing diethanolamine and thiourea, that was prepared according to the method of Pardee.[11, 12] This "CO_2 buffer" maintained a constant gas phase of 5 per cent CO_2 and 95 per cent O_2 during measurements of oxygen consumption when the side wells were open to the main chamber.

Tissue samples of closely similar wet weights, as determined by weighing on a Roller-Smith torsion balance, were suspended in 1.5 ml. of Krebs-Ringer-bicarbonate solution in each flask. Tetracyclene hydrochloride (1:100,000) was added to the suspending fluid to inhibit bacterial growth. Equilibration of CO_2 in the gas phase was expedited by flushing and filling the flask-manometer systems initially with the desired gas mixture of O_2 95 per cent–CO_2 5 per cent. Initial equilibration proceeded for 25 minutes while the flask-manometer systems were shaken in the conventional Warburg apparatus, with the water bath set at 38° C. Oxygen consumption was then measured for a period of 30 minutes with CO_2 absorption taking place in the buffer in the open side wells. At the end of that time the manometers were read, the side wells were closed, and the following 30 minutes were devoted to measurement of carbon dioxide production. After the manometer changes for this measurement had been recorded, the side wells were opened, and the systems were equilibrated for 10 minutes. This was followed by the next cycle of measurements, each of which required approximately 75 minutes for completion.

A minimum of three flask-manometer systems loaded with solutions identical with those of the other systems, but without tissue, were used for thermobarometers, and the TBM correction was the average of their readings. Gas volumes were measured by Lazarow's method.[13] Two gas-volume determinations were required for each system; one with the side wells open and another with the side arms closed. This was necessitated by different flask constants in calculating T_{O_2} and T_{CO_2}. When using the Stanley-Tracewell respiratory quotient flasks, it is possible to obtain the R.Q. simply by dividing T_{CO_2} by T_{O_2}, but in this study both the Q_{O_2} and the Q_{CO2} were

calculated to provide a check on the consistency of tissue behavior with that previously observed. Both these quotients were calculated on a dry tissue-weight basis.

Twelve samples of embryonic and 12 samples of young bovine dental pulp were studied to determine the rates of oxygen consumption and carbon dioxide production during alternating 30-minute periods through 9 complete cycles. The measurements for any sample, therefore, extended through a span of about 11 hours. About 2½ hours elapsed between the time the animal was killed and measurements were started. Thus the last measurements were made approximately 13½ hours after the pulp tissue had been deprived of an active circulation. Five samples of mature dental pulp were similarly studied, except that measurements were completed through only 6 complete cycles. The measurements for any of these samples extended through about

TABLE 1*

ENDOGENOUS RESPIRATION AT 38° C. IN BOVINE PULP FROM TEETH IN VARIOUS STAGES OF DEVELOPMENT

(Measured over Pardee Buffer)

	75-MINUTE PERIODS								
	1	2	3	4	5	6	7	8	9
12 samples—embryonic pulp									
Q_{O_2}	3.18	3.56	3.22	2.96	2.90	2.74	2.44	2.78	2.10
Q_{CO_2}	2.74	2.98	2.36	2.12	1.84	2.00	1.68	1.96	1.30
R.Q.	0.86	0.84	0.74	0.74	0.63	0.73	0.68	0.71	0.61
S.D.-R.Q.	0.09	0.08	0.05	0.05	0.07	0.15	0.07	0.13	0.13
12 samples—young pulp									
Q_{O_2}	1.30	1.62	1.54	1.64	1.79	1.82	1.75	1.82	1.67
Q_{CO_2}	1.22	1.36	1.35	0.94	1.22	1.25	1.24	1.36	1.08
R.Q.	0.94	0.84	0.88	0.57	0.68	0.69	0.71	0.75	0.65
S.D.-R.Q.	0.13	0.22	0.10	0.08	0.14	0.09	0.10	0.07	0.11
5 samples—mature pulp									
Q_{O_2}	0.48	0.56	0.46	0.62	0.66	0.86	.....	.....	.....
Q_{CO_2}	0.68	0.56	0.68	0.58	0.50	0.64	.....	.....	.....
R.Q.	1.42	1.00	1.48	0.94	0.76	0.74	.....	.....	.....
S.D.-R.Q.	0.26	0.15	0.15	0.20	0.29	0.07	.....	.....	.....

* Released CO_2 is sum of respiratory CO_2 and acid production.

7½ hours, and the last measurements were made approximately 10 hours after the animals were killed.

RESULTS

The measurements of oxygen consumption and carbon dioxide production on these 29 pulp samples permitted calculation of 246 oxygen quotients, 246 carbon dioxide quotients, and 246 respiratory quotients. The mean values for these three quotients are presented in Table 1.

It will be noted that the mean respiratory quotient calculated from measurements of embryonic pulp tissue during the first period lay in the range usually assigned for protein metabolism, while that for young pulp was probably indicative of carbohydrate metabolism. The respiratory quotient for mature pulp during the same period was well above the value ascribed for carbohydrate metabolism, if only respiratory

carbon dioxide were involved. Hence it must be assumed that considerable acid production was occurring at the same time in this tissue.

During the second, third, and fourth periods, both embryonic and young pulp showed a progressive decline in carbon dioxide production relative to a reasonably stable rate of oxygen consumption, suggestive of a shift toward protein and lipid metabolism. Thereafter, their respiratory quotients remained close to 0.7. The mature pulp tissue also showed a fairly constant, although considerably lower, rate of oxygen consumption during these same periods, but the respiratory quotient fluctuated rather widely. Nevertheless, the character of the respiratory quotient is suggestive of carbohydrate metabolism through the fourth period. After the fourth period the mature pulp produced carbon dioxide in relation to oxygen consumed in a ratio indicating a shift toward protein and then lipid metabolism. By the sixth period its respiratory quotient was approximately the same as those of the other two classes of pulp tissue (Fig. 1).

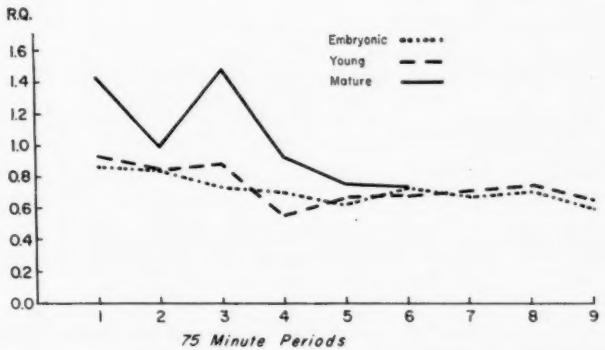

FIG. 1.—Respiratory quotients of bovine dental pulp—38° C.

When these facts became apparent, a brief supplementary experiment was conducted to determine the respiratory quotient of young pulp tissue based on the measurement of only respiratory carbon dioxide production from a substrate of 0.2 per cent glucose. The suspending fluid was Krebs-Ringer-phosphate solution to which the glucose was added. The CO_2 absorbent in the side wells was KOH solution. This experiment was conducted with 5 samples of tissue through only 2 cycles of determinations, in order to avoid significant pH changes that would make calculations of CO_2 retention extremely difficult or impossible. One sample was withdrawn at the end of the first cycle of oxygen consumption and carbon dioxide production measurements for the purpose of checking the pH change of the suspending fluid. The respiratory quotients derived from this experiment are presented in Table 2. The Q_{CO_2} values on which they are based were obtained by manometric measurement, plus the calculated retention of carbon dioxide in the buffered suspending fluid.

These results show that when glucose is available for the metabolic requirements of at least young pulp tissue and that when only respiratory carbon dioxide production

is measured, the value of the respiratory quotient falls within the range usually associated with carbohydrate metabolism.

DISCUSSION

This and a previous study have shown that the metabolic activity of the dental pulp, as indicated by its rate of oxygen uptake, is generally proportional to its level of dentinogenetic activity. This study has also shown that young pulp tissue has a respiratory quotient characteristic of carbohydrate metabolism when glucose is available to it. The consistency of the respiratory quotient of young pulp tissue samples provided with glucose, as contrasted with declining R.Q. of pulp tissue of similar metabolic activity but to which glucose was denied, strongly suggests that the behavior of the latter is attributable to exhaustion of the tissue's supply of available carbohydrate. Where the young pulp was not supplied with glucose, enough available carbohydrate remained in the tissue at the end of approximately 3½ hours after deprivation of circulation to be productive of a mean R.Q. of 0.94. Slightly more than an hour later there was no evidence of predominant carbohydrate metabolism.

TABLE 2*

RESPIRATORY QUOTIENTS OF BOVINE YOUNG DENTAL PULP WITH CARBOHYDRATE SUBSTRATE

	SAMPLE					MEAN R.Q.	S.D.
	A	B	C	D	E		
R.Q., 1st hr.....	0.99	0.84	0.96	0.91	0.95	0.93	0.05
R.Q., 2d hr.....	0.97	0.86	0.94	0.95	.....	0.93	0.04

* Q_{O_2} and Q_{CO_2} determined at 38° C. and calculated on dry-weight basis. Substrate: Krebs-Ringer-phosphate solution with 0.2 per cent glucose.

The embryonic pulp tissue of this study was more than twice as active as the young pulp, as shown by their respective oxygen quotients. At the end of the first complete cycle of measurements, 3½ hours after the tissue was deprived of an active circulation, the mean respiratory quotient of 0.86 points to the possibility that this more active tissue had already exhausted its carbohydrate reserves. It would seem that its respiration was being sustained on protein constituents.

The mature pulp is presumed to be the least active of the three varieties studied, exhibiting an oxygen consumption rate of about one-third that of the young pulp tissue. The slower metabolic rate apparently demanded smaller quantities of carbohydrate per unit of time, since its mean R.Q. dropped to 0.94, still within the carbohydrate range, only after the fourth period of measurement and about 7½ hours after the tissue had been separated from its circulation. Slightly more than an hour later, it, too, failed to show the characteristics of carbohydrate metabolism.

All these findings point quite suggestively to carbohydrate metabolism and probably that of glucose as an important aspect of the respiratory process in dental pulp. Continued survival of this tissue after exhaustion of its seemingly limited glucose reserves appears to be at the expense of its protein and lipid constituents.

A point of interest with respect to mature pulp tissue is its possible capacity for acid production. Reference to Table 1 and Figure 1 will show that at the end of the first and third periods of measurement, while glucose is presumed to be still present in the tissue, the R.Q. values are greater than 1.4. Table 2 shows that the R.Q. of tissue supplied with glucose and measured only for respiratory carbon dioxide is around 0.93. This leads one to suspect that the greater portion of the R.Q. value above 1.0 is attributable to the production of acid that displaces carbon dioxide from the Krebs-Ringer-bicarbonate solution. If this is true, it may possibly indicate a significant glycolytic capacity of dental pulp. Should this be substantiated, many of the frequently observed and presently unexplained fluctuations in the rate of oxygen consumption that occur in samples of pulp tissue studied over a period of several hours may eventually be traced to metabolic vacillations between aerobic and anaerobic pathways.

SUMMARY

Respiratory quotients were determined for several samples of dentinogenetically very active, moderately active, and relatively quiescent bovine dental pulp that showed proportionate levels of oxygen consumption. These determinations were repeated at approximately 75-minute intervals for a minimum of 6 periods in the instance of the most quiescent tissue. The respiratory quotients suggest that carbohydrate reserves in the pulp are limited and are rapidly exhausted in the more active tissue. Approximately $8\frac{1}{2}$ hours after the least active tissue was deprived of circulation it failed to show evidence of carbohydrate metabolism and, as the more active tissues had shown earlier, was presumed to be supporting respiration by oxidation of its protein and lipid constituents. It is suspected that the dental pulp has a significant glycolytic capacity because of indirect evidence of acid production.

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The Importance of the Genotype on Susceptibility to Dental Caries in the Rat

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In 1944, Hunt, Hoppert, and Erwin[1] demonstrated the inheritance of susceptibility to caries in albino rats. By use of selection, progeny testing, and inbreeding, caries-resistant and caries-susceptible lines of rats were developed from a group of heterogeneous animals.

Keyes[2] has shown that caries can be induced in caries-inactive animals by exposure to caries-active animals. His results indicated that "resistant" animals do not develop caries because the "cariogenic" oral micro-organisms are absent.

This paper presents data which show that the caries-resistant and -susceptible lines of rats originally developed by Hunt *et al.*[1] retained their characteristic behavior with respect to caries when exposed to the oral environment of the opposite strain.

MATERIALS AND METHODS

On the day of birth, we placed entire litters of caries-resistant rats with susceptible mothers and entire litters of caries-susceptible rats with resistant mothers. Subsequent litters from the same matings were kept with their natural mothers and served as controls. The rats were given a modified Hoppert-Webber-Canniff coarse-particle caries-test diet with weekly supplements of raw beef liver. Examinations for gross occlusal fissure lesions were made in the live rat by macroscopic observation of the mandibular molars every 2 weeks. The age in days at which the rat developed caries was designated the "caries age." Details of this procedure for estimating caries activity have been described previously.[1]

The caries-susceptible rats used in this experiment were actually OMS rats developed by us several years ago by mating Osborne-Mendel rats with our original caries-susceptible line (unpublished data). The OMS rats are now as extremely and uniformly susceptible to caries as our original susceptibles.

RESULTS

The results are given in Table 1. Caries-susceptible rats which remained with their natural mothers developed caries at 65 ± 2.3 days of age. Caries-susceptible rats obtained from the same matings but fostered by a caries-resistant mother developed caries at 64 ± 1.2 days of age. Obviously, there was no significant difference in the rate of

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caries development in susceptible rats when nursed by their natural mother or by a caries-resistant mother. The caries age for resistant rats nursed by their natural mothers was 297 ± 15 days, whereas the resistants which were fostered by susceptible mothers developed caries in 341 ± 17 days. The difference of 44 days between the latter two experimental groups was not quite significant at the 5 per cent confidence level, although it was surprising that resistant rats fostered by susceptibles developed caries later than did resistants nursed by their natural mothers.

TABLE 1

EFFECT ON CARIES OF FOSTERING CARIES-RESISTANT RATS WITH SUSCEPTIBLES AND VICE VERSA

Newborn Strain	Nurse Strain	No. of Rats	Caries Age (Days)	
			Mean and Standard Error	Range
Susceptible	Susceptible	38	65±2.3	45–111
Susceptible	Resistant	45	64±1.2	55– 84
Resistant	Resistant	37	297±15	176–535
Resistant	Susceptible	39	341±17	164–651

DISCUSSION

These results prove that, under the specific conditions of this experiment, the genetic constitution of an animal is much more important than exposure to the oral environment of the opposite strain. The response to caries of the rats which were fostered by a caries-resistant mother was identical with that of susceptible rats nursed by their natural mother. There was also no significant difference in caries incidence between the resistant rats which were nursed by susceptible or resistant mothers. In a similar experiment, Shaw[3] reported that the Harvard strains of caries-resistant and caries-susceptible rats showed the caries activity expected from their genetic background when fostered by mothers of the opposite strain. In Shaw's experient a high-sucrose, instead of the coarse-particle, caries-test diet was used. Thus two different groups of investigators working with different strains of rats and different caries-test diets have now reported results reaffirming the earlier work of Hunt *et al.*[1] These results unquestionably show that genes influence an animal's resistance to caries.

Keyes[2] has shown that caries could be transmitted in Osborne-Mendel rats. When he administered penicillin to young rats for a short period of time and the rats were then caged with caries-active rats which had not been given penicillin, they became caries-active. Littermates that were also given penicillin for a short period of time but were not caged with caries-active rats remained caries-inactive. Current experiments in our laboratory confirm this finding. However, we have given penicillin from birth to weaning and then have inoculated these rats with feces from both caries-resistant and caries-susceptible (OMS) rats. We find that caries-susceptible rats treated in this manner show greater caries activity when inoculated with feces from either strain of rat than littermate susceptibles which were also given penicillin during the suckling period but were not inoculated with feces from either resistant or susceptible rats. These results will be submitted

for publication subsequently. These studies suggest that our caries-resistant strain of rats harbors a caries-inducing flora. The resistance of this line of rats would then be due either to the failure of the caries-inducing micro-organisms to establish themselves in sufficient quantity and/or to hereditary influences whose precise nature is as yet unknown, which confer caries resistance on this particular group of animals. The physiologic and other effects of genes responsible for the susceptibility and/or resistance to caries is apparently complex. Hunt, Hoppert, and Rosen[4] have indicated the interrelationships of certain factors responsible for susceptibility to caries. Tooth morphology and composition, saliva, systemic factors, etc., may all influence the production of caries.

Keyes[2] has shown that hamsters which were caries-inactive could become caries-active when caged with other caries-active hamsters. He could not have used caries-inactive hamsters that were analogous to our caries-resistant rats. No genetically caries-resistant line of hamsters has yet been developed. If hamsters as a species do not possess genes which can influence resistance to caries, it would be impossible to establish a genetically caries-resistant line. A geneticist should be encouraged to attempt to develop a genetically caries-resistant stock of hamsters.

What is a satisfactory caries-resistant line of animals? Ideally, a caries-resistant stock should never develop a single carious lesion under any dietary regimen in any generation. Such "absolute resistance" has never been attained by breeding; and resistance can be at best only relative. A resistant line of animals should be compared with a line that is susceptible. If the two lines can be distinguished with confidence, then one line may be termed susceptible and the other resistant. The caries-resistant rats used in the present study were less resistant than the rats used in previous studies.[1, 4] Nevertheless, the difference between the two lines is very obvious.

SUMMARY

Newly born caries-resistant rats were fostered by caries-susceptible mothers and vice versa. The caries-resistant young remained caries-resistant; the caries-susceptible young remained susceptible. The foster mothers did not affect the caries experience of the young they nursed. It may be concluded that, in these animals, genotype played a role in their resistance or susceptibility to dental caries.

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Nitrate Reduction in Whole Saliva

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The importance of the composition of saliva as a factor influencing dental caries has repeatedly attracted the attention of investigators studying this disease.[1, 2] Since the demonstration that dental caries is unquestionably of an infectious nature,[3] interest in salivary components that serve as essential metabolites and growth factors for members of the oral flora is especially apropos. Such contributing compounds as amino acids and vitamins have been identified in saliva.[4–10] However, to date, the metabolism of inorganic nitrogen in saliva (by the oral flora) has received little attention. The fact that both nitrate[11] and ammonia[12]—nitrogen in its most reduced state and assumed to be the final product in the reduction of nitrate[13]—have been reported to occur in saliva suggests that the reduction of salivary nitrate might play a role in bacterial growth in the oral cavity. It is generally believed that inorganic nitrogen, only in the form of ammonia, can be synthesized into organic nitrogen compounds;[14] hence the reduction of nitrate by saliva may possibly lead to the assimilation of nitrogen. At the same time it is possible that alternately, or in addition, nitrate and/or one of its reduction compounds may serve as hydrogen acceptors in reactions concerned with the energy metabolism[15] of certain oral bacteria. On the other hand, salivary nitrate may act as a hydrogen acceptor in place of a usual substance and thereby interfere with a normal energy yielding reaction[16] of the oral flora.

This paper describes some preliminary observations of the reduction of endogenous and added nitrate by whole saliva, as well as the presumptive reduction of the resulting nitrite. However, the reduction product(s) of nitrite have not, at this time, been identified in saliva.

EXPERIMENTAL METHODS

The saliva examined in this study was obtained by paraffin stimulation and was collected in test tubes immersed in crushed ice. The saliva was maintained in the ice bath until it was used, this period usually being less than 1 hour.

The nitrate in saliva was determined by the method described by Whelan[17] and was deproteinized for this analysis according to this author's recommendation for plasma, serum, and ascitic fluid. The method is based on the oxidation of colorless diphenylbenzidine by nitric or nitrous acid to its intensely blue quinoidimonium salt. Although nitrite, as well as nitrate, gives the characteristic blue color of oxidized diphenylbenzidine, the method is quantitative for both nitrate and nitrite. Parallel nitrite analyses were made with each nitrate determination whenever absolute values for nitrate were desired.

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Standard nitrate solutions (KNO_3) were prepared in the range of 0.2–1.0 μg. nitrate per milliliter, and the concentration that gave a blue color of optical density closest to 0.4 (at 600 mμ determined with a Bausch and Lomb Spectronic 20 colorimeter) was used as a standard for comparison with the saliva dilution that most closely approximated the same color development. This comparison procedure was recommended by Whelen.[17] It was found more satisfactory on the basis of good duplicate determination on salivary samples at different dilutions than comparison of the unknown with a series of standards. Also, the complete recovery of nitrate added to saliva was observed. It was usually found that an initial dilution of saliva between 1:5 and 1:20 provided a nitrate concentration at a level necessary for the technique.

Although this technique per se appeared to be satisfactory for the measurement of nitrate in saliva, the necessity of using sulfuric acid free of nitrate complicates the use of the method. As a result of the difficulty in obtaining sufficiently pure sulfuric acid, only a limited number of satisfactory nitrate determinations were performed. However, since the determination of salivary nitrite is more easily and more rapidly accomplished than the assay of salivary nitrate, nitrate reduction has, for the most part, been described and illustrated throughout this paper in terms of nitrite production and/or accumulation instead of nitrate disappearance. This would seem to be a valid assertion, for there is general agreement that nitrite is the first product in the stepwise reduction of nitrate.[18]

Before salivary nitrite was determined, saliva was freed of coagulable proteins by alkaline precipitation with zinc sulfate, essentially as outlined by Somogyi for blood and modified by Steigletz and Palmer.[19] Two ml. of fresh saliva were placed in 12-ml. centrifuge tubes containing 5 ml. of a 4.5 per cent solution of zinc sulfate and were thoroughly mixed. One ml. of 1 N sodium hydroxide was then added, followed again by thorough mixing. The tubes were centrifuged for 20 minutes at 2000 r.p.m., and the supernatant was decanted into another centrifuge tube for an additional centrifugation. It was usually necessary to centrifuge the supernatant a second time to insure an absolutely clear supernatant.

The nitrite concentration in the deproteinized supernatant was quantitatively determined colorimetrically, using sulfanilic acid and α-naphthylamine, as originally suggested by Ilosvay and described by Stickland.[20] The technique depends on the diazotization of sulfanilic acid by nitrous acid, followed by coupling with α-naphthylamine to form a red azo dye. The reagents were prepared in a manner similar to that described by Csaky.[21] One and two-tenths gm. of sulfanilic acid were dissolved in 150 ml. of 5 N acetic acid, and 0.75 gm. of α-naphthylamine was also placed in another 150-ml. aliquot of 5 N acetic acid. Although the α-naphthylamine is not completely soluble at this concentration, no attempt was made to remove the residue. Care was taken to draw off only the solution when compounding the color reagent. The two solutions were stored in individual brown bottles and mixed in a proportion of 1:1, 5 minutes before use.

One-ml. aliquots of the deproteinized saliva were placed in 105 × 10-mm. cuvettes, and 0.2 ml. of the 1:1 mixture of sulfanilic acid and α-naphthylamine reagent was added. The volume was adjusted to 5 ml. with double-distilled, deionized water, and the optical density of the red solution of azo dye was measured at 520 mμ in a Bausch and Lomb Spectronic 20 colorimeter. The optical densities recorded for salivary

specimens were compared with that obtained for standard solutions of sodium nitrite. Satisfactory recoveries of nitrite ($NaNO_2$) added to salivary specimens before deproteinization were obtained, as well as good duplicate reproduction on salivary samples at different dilutions.

The course of nitrate reduction was followed by incubating 30 ml. of saliva in 25 × 200-mm. test tubes in a constant-temperature water bath at 37° C. Periodically, aliquots were removed from the incubating sample, and the progressive changes in the nitrate and/or nitrite concentration were determined.

To characterize quantitatively the capacity of a salivary sample to reduce nitrate through nitrite, the sample was initially depleted of endogenous nitrate and nitrite by incubation at 37° C. until nitrite was no longer detectable in the sample. This usually required less than 2 hours. Five ml. of the nitrate-nitrite-depleted saliva were transferred to 15 × 100-mm. test tubes in a constant-temperature water bath at 37° C. (To establish standard conditions, the same size tubes were always used for this incubation, to maintain a constant surface-to-volume ratio, since oxygen inhibits the reduction of nitrite by the enzyme systems in saliva.) Fifty μg. of nitrate (KNO_3) in 0.1 ml. of solution were added to the incubating saliva, and the time required to reduce the added nitrate through nitrite was determined.

The rate of nitrate reduction, through nitrite, was followed by removing aliquots from the incubating saliva-nitrate mixture, dropwise, at 5-minute intervals and placing the drops in the depression of a spot plate containing 0.2 ml. of the sulfanilic acid–α-naphthylamine reagent. The (red) color developed could readily be compared with that of the color reagent in adjacent depressions. The time required for the complete disappearance of nitrite from the incubating saliva, as indicated by the failure of color development, was readily determined. Employing the results of this technique, the nitrate-reducing activity of saliva can be expressed as the micrograms of nitrate reduced through nitrite per milliliter of saliva per minute. By using a drop of saliva for each of the serial determinations, the assay of relatively small volumes of saliva (2.5–5 ml.) can be accomplished.

RESULTS

Reduction of nitrate.—When whole saliva was incubated at 37° C. and its nitrate and nitrite levels determined periodically, the nitrate concentration was observed to decrease progressively. The data presented in Figure 1 illustrate the relatively rapid loss of nitrate from an incubating sample of saliva. Although about 28 μg. of nitrate per milliliter were initially present in this particular specimen, nitrate was no longer detectable after 75 minutes.

During the early period of incubation, when the nitrate level of the incubating saliva was decreasing, a transient accumulation of nitrite was observed. In the case of the specimen illustrated in Figure 1, the nitrite concentration increased approximately 55 per cent in the first 45 minutes of incubation and subsequently decreased to zero in an additional 60 minutes.

It was further found that the addition of nitrate (KNO_3) to saliva was followed by its prompt reduction. This was indicated by the greater magnitude of nitrite accumulation and its rate of disappearance in saliva to which nitrate was added, as compared with an aliquot of the same saliva containing only endogenous nitrate and

nitrite (sample *A*, Fig. 2). In addition, it was also observed that saliva depleted of its endogenous nitrate and nitrite by prior incubation will rapidly reduce added nitrate (sample *B*, Fig. 2).

Separation of the active components of the salivary nitrate-reducing system.—When a sample of nitrate-nitrite-depleted saliva was centrifuged at 10,000 r.p.m. for 30 minutes, neither the supernatant nor the resuspended sediment, washed in saline, distilled water, or phosphate buffer at pH 6, demonstrated a capacity to reduce added nitrate or nitrite ($NaNO_2$). However, the recombination of supernatant and sediment restored this reductase activity, although it was always somewhat less than that of the whole saliva from which it was derived (Table 1). In addition, it was observed

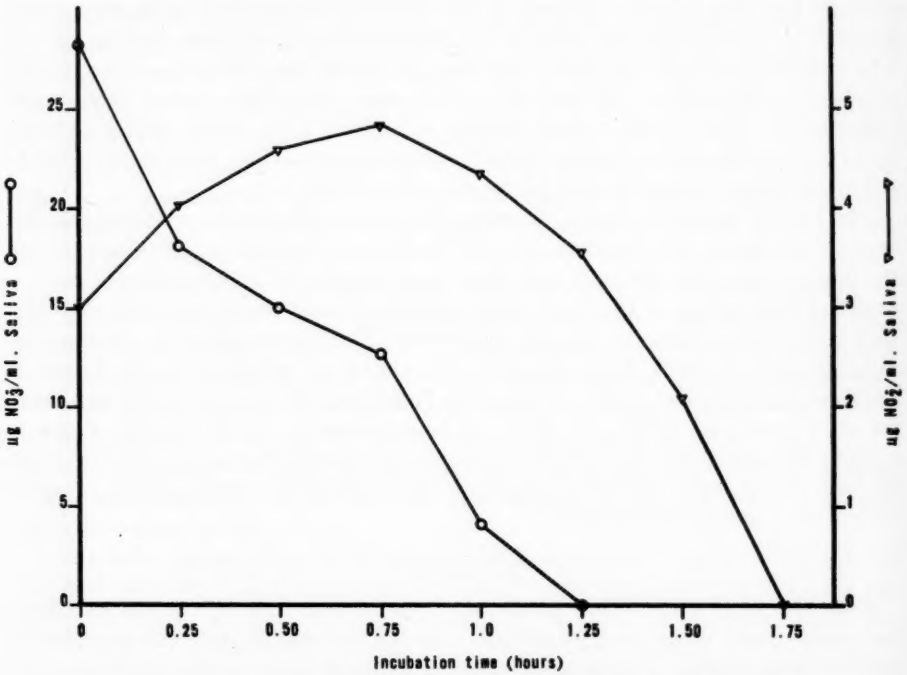

FIG. 1.—Progress changes in the endogenous NO_3^- and NO_2^- levels in incubating saliva

that washing the sediment in saline, distilled water, or the phosphate buffer one, two, or three times resulted in only a slight decrease in the reductase activity of the recombination of sediment that was washed three times and its supernatant (Table 2).

It was also observed that dialyzing saliva for 24 hours against six times its volume of distilled water did not affect its capacity for reducing either nitrate or nitrite.

The observation that neither the supernatant nor the sediment alone would reduce nitrate or nitrite suggested that the appropriate enzyme(s) was located in one of the fractions and that the other provided the necessary elements of an electron donor system. In order to determine this possibility, nitrate and a potential hydrogen donor were added to the supernatant and resuspended sediment from nitrate-nitrite-depleted

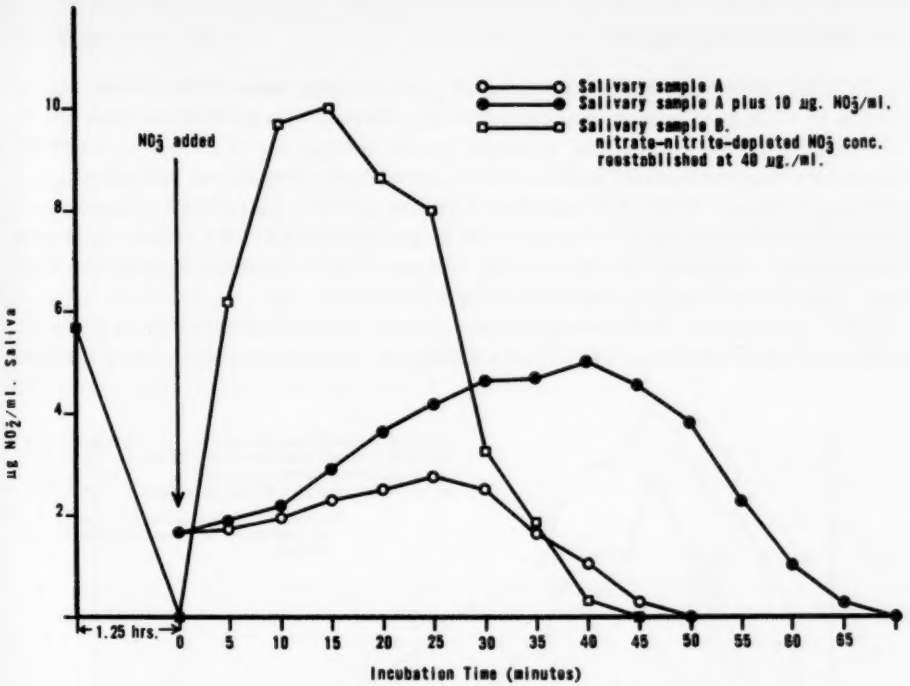

FIG. 2.—Progressive reduction of added nitrate (KNO_3^-), through nitrite, in saliva and in nitrate-nitrite-depleted saliva.

TABLE 1

COMPARISON OF NITRATE-REDUCING CAPACITY OF WHOLE SALIVA WITH RECOMBINATION OF SUPERNATANT AND SEDIMENT FROM SAME SAMPLE, SHOWING LOSS IN ACTIVITY RESULTING FROM TEMPORARY SEPARATION OF FRACTIONS
(μg. NO_3^- Reduced through NO_2^-/Ml/Min)

	Sample				
	1	2	3	4	5
Whole saliva.....	0.20	0.17	0.28	0.25	0.28
Supernatant plus sediment...	0.17	0.12	0.25	0.20	0.22

TABLE 2

COMPARISON OF REDUCING CAPACITY OF WHOLE SALIVA WITH THAT OF RECOMBINATION OF ITS WASHED AND UNWASHED SEDIMENT PLUS SUPERNATANT
(μg. NO_3^- Reduced through NO_2^-/ml of Reaction Mixture per Minute)

Whole Saliva	Supernatant plus Sediment: Number of Times Sediment Washed in									
	Buffer				Saline			Water		
	0	1	2	3	1	2	3	1	2	3
0.17.....	0.12	0.12	0.12	0.11	0.12	0.12	0.09	0.12	0.12	0.08

saliva and buffered at pH 6.2. When lactate, pyruvate, or yeast extract, at a concentration of 1.0 mg/ml, was substituted for the supernatant as a possible electron donor, resuspended sediment was found to reduce nitrate through nitrite, but at a somewhat slower rate than recombined sediment and supernatant. Formate was only effective as a hydrogen donor for nitrate reduction. Cysteine, cystine, glutathione, glucose, succinate, and thioctic acid were not effective as hydrogen donors for either the nitrate or the nitrite reductase systems in the sedimnet. None of these compounds, plus supernatant, would reduce either nitrate or nitrite.

Effect of filtering, boiling, and autoclaving on the nitrate-reducing activity of saliva.—Passing through a Seitz filter, boiling, or autoclaving saliva for 5 minutes

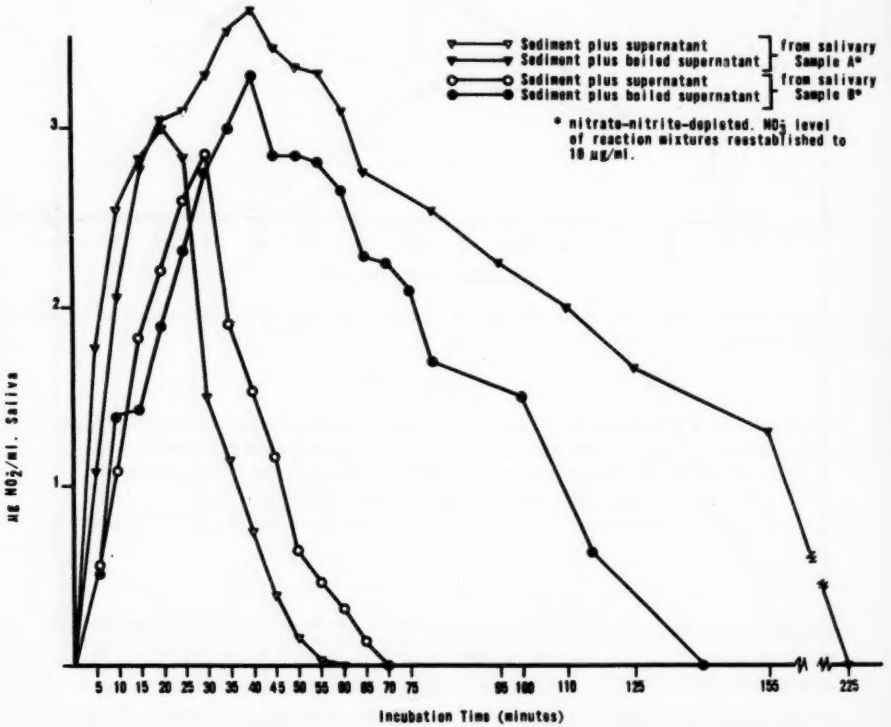
Sediment plus supernatant
Sediment plus boiled supernatant
from salivary Sample A*
Sediment plus supernatant
Sediment plus boiled supernatant
from salivary Sample B*
* nitrate-nitrite-depleted. NO3 level of reaction mixtures reestablished to 10 µg/ml.
µg NO2/ml. Saliva
Incubation Time (minutes)

FIG. 3.—Nitrate-nitrite reduction by sediment plus supernatant compared with sediment plus boiled supernatant.

completely destroys its capacity to reduce nitrate or nitrite. However, the recombination of boiled supernatant with untreated sediment was found to reduce nitrate to nitrite at a rate approximately equivalent to that of sediment recombined with supernatant that had not been heated (Fig. 3). On the other hand, this combination of sediment plus boiled supernatant showed a marked loss of capacity to reduce nitrite, requiring from two to three times longer for the complete reduction of 10 μg. of nitrate/ml through nitrite. This was in contrast to the combination of boiled sediment plus unheated supernatant that would not initiate the reduction of either nitrate or nitrite.

The addition of lactate, pyruvate, or yeast extract (1 mg/ml) to the combination of sediment plus boiled supernatant restored the capacity of this system to reduce nitrite.

Optimum pH for nitrate reduction by saliva.—The effect of hydrogen-ion concentration on the capacity of saliva to reduce nitrate through nitrite was determined in aliquots, from a common pool of nitrate-nitrite-depleted saliva, the pH of which was adjusted with phosphate buffer. Maximum activity was found to be demonstrated between pH 6 and 6.4, with marked and progressive decreases on either side of these relatively narrow limits (Fig. 4, *A*). At pH 4 and below and at pH 9 and above, no reduction of added nitrate was apparent. Between pH 8.2 and 8.6, nitrate is reduced to nitrite, but nitrite reduction is inhibited (Fig. 4, *A* and *B*).

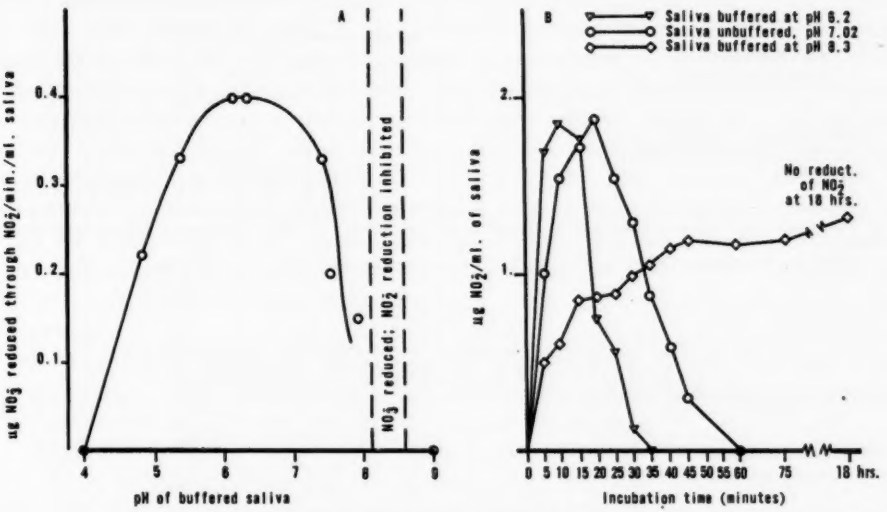

FIG. 4.—Effect of pH on the reduction of nitrate by whole saliva

Relationship between the amount of sediment in saliva and its nitrate-reducing capacity.—Ten ml. samples of saliva were collected from 40 young adults, and the nitrate-reducing capacity of each was determined. The amount of sediment per milliliter of each sample was estimated by filling 1-ml. pipettes, securing them in an upright position for 2 hours, and observing the volume of sediment that collected in the tip. It was found, by this crude technique, that there was a relatively low positive correlation ($r = 0.31$; $P = 0.05$) between the amount of sediment per milliliter of saliva and its nitrate-reducing capacity.

In addition, the effect of varying the amount of sediment in a sample of saliva on its nitrate-reducing activity was determined by recombining the supernatant from 5 ml. of saliva with varying amounts of sediment from the same salivary sample. The reducing activity was found to increase as the amount of sediment in the reaction mixture was increased (Table 3). The same relationship was found between the nitrate-reducing activity and the amount of supernatant (electron donor) combined with a constant amount of sediment (Table 4).

The influence of oxygen on nitrate and nitrite reduction by saliva.—The effect of oxygen on nitrate-nitrite reduction by incubating saliva was demonstrated by aerating two 30-ml. aliquots of saliva from a common salivary pool. The saliva was initially depleted of endogenous nitrate and nitrite, and the nitrate concentration was re-established at 10 μg/ml in both aliquots. During incubation the change in nitrite concentration in each aliquot was determined as air was passed through one and nitrogen washed in a pyrogallol solution was passed through the other. It was apparent, on the basis of these experiments, that nitrate reduction to nitrite by whole saliva occurs

TABLE 3

EFFECT OF INCREASING AMOUNTS OF SEDIMENT RECOMBINED WITH SUPERNATANT ON NITRATE-REDUCING ACTIVITY OF SALIVA

PLUS SUPERNATANT* FROM 5 ML. SALIVA	SEDIMENT* FROM ML. OF SALIVA										
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	10
μg NO_3^- reduced through NO_2^- per ml. of reaction mixture per minute	†	†	†	†	0.11	0.11	0.12	0.12	0.13	0.17	0.29

* Supernatant and sediment from same nitrate-nitrite-depleted salivary specimen. NO_3^- concentration re-established at 10 μg/ml.

† NO_3^- reduced relatively rapidly; resulting NO_2^- not completely reduced in 3 hours and 15 minutes.

TABLE 4

EFFECT OF INCREASING AMOUNTS OF SUPERNATANT RECOMBINED WITH SEDIMENT ON NITRATE-REDUCING ACTIVITY OF SALIVA

PLUS SEDIMENT* FROM 5 ML. SALIVA	SUPERNATANT* FROM ML. OF SALIVA									
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
μg NO_3^- reduced through NO_2^- of reaction mixture per minute	0.09	0.10	0.10	0.12	0.12	0.13	0.14	0.15	0.18	0.22

* Supernatant and sediment from same nitrate-nitrite-depleted salivary specimen. NO_3^- concentration re-established at 10 μg/ml.

under either aerobic or anaerobic conditions. However, the presence of oxygen completely inhibits the reduction of nitrite.

It was subsequently observed that the degree of anaerobiosis that developed while incubating saliva in test tubes was sufficient for the reduction of nitrite and that gassing with nitrogen did not increase the reduction rate.

Lack of relationship between capacity of saliva to reduce nitrate and caries experience.—Ten ml. of paraffin-stimulated saliva was collected from 40 young adults whose ages ranged from eighteen to twenty-six years. The capacity of these salivary samples to reduce nitrate through nitrite was determined. No significant correlation between the individual's DMF and μg. of nitrate reduced per minute per milliliter by his saliva was found ($r = 0.11$; $P = 0.47$).

DISCUSSION

That the reduction of nitrate and the apparent reduction of nitrite in saliva are enzyme-catalyzed reactions is suggested by the effects of heat, hydrogen-ion concentration, and the presence or absence of electron donors.

The enzymes that participate in the reduction of nitrate and nitrite are apparently present in the particulate fraction of saliva. Although neither sediment nor supernatant alone will reduce nitrate or nitrite, when a hydrogen donor substance, such as yeast extract, pyruvate, or lactate is added to sediment, the mixture will reduce both nitrate and nitrite. This is in contrast to the supernatant when it is allowed to act on nitrate or nitrite in the presence of such hydrogen donors as yeast extract, succinate, formate, lactate, pyruvate, cysteine, cystine, glutathione, glucose, or thioctic acid, neither nitrate nor nitrite is reduced.

Also, even though the measure of correlation between the nitrate-nitrite-reducing capacity of saliva and its content of sediment was relatively low ($r = 0.31$) and on the border line of significance, the other findings relative to the effects of the presence or absence of sediment, as well as varying the amount of sediment in recombined saliva, confirmed that the value and sign obtained for the correlation coefficient was a true expression of the relationship.

Further corroborative evidence that these enzyme systems are in the sediment and that the necessary hydrogen donors are supplied by the supernatant was found in the observation that boiled sediment plus pyruvate, lactate, or yeast extract or untreated supernatant would not reduce either nitrate or nitrite. However, sediment plus boiled supernatant would reduce nitrate through nitrite, although the rate of nitrite reduction was markedly decreased. Apparently, the electron donor in the supernatant that normally reacts with the nitrite reductase in sediment is partially destroyed or altered by boiling, but not the one necessary for nitrate reduction.

The observation that sediment in the presence of boiled supernatant will reduce nitrate at an unaltered rate but that the rate of nitrite reduction is decreased along with the finding that nitrite reduction by whole saliva in the presence of oxygen will proceed only as far as nitrite suggests that salivary nitrate and nitrite reductase are two distinct enzyme systems and may be quite different.

A preliminary attempt to identify the metals of the nitrate and nitrite reductase systems in saliva was not successful, since the dialysis of saliva against distilled water or the thorough washing of its sediment failed to inactivate the enzymes. The metals, if any, seem to be firmly bound to the enzyme protein.

It is difficult to draw any specific conclusions about the physiologic significance of the data presented in this paper, since the end product(s) of nitrate reduction by saliva are, as yet, unknown. However, the reduction of salivary nitrate in the presence of oxygen presumably does not play a role in the assimilation of nitrogen, since it does not proceed past the nitrite stage. Also, an explanation of the import of the aerobic reduction is tenuous on the basis of nitrate serving as an oxidant for cellular energy metabolism in the presence of oxygen. This is in contrast with the anaerobic reduction of nitrate, where nitrite might be serving as a terminal oxidant for important reduced intermediates formed by oxidation processes. Nitrate reduction would, thereby, possibly be of importance energetically to certain oral micro-organisms under

some conditions. Also, if work now in progress demonstrates that ammonia is a product of this anaerobic reductive process in saliva—and most workers assume that it is the final product of nitrate reduction[13]—the metabolic function of the reduction of nitrate to nitrite and further to ammonia would appear to be a source of nitrogen for the synthesis of organic nitrogen compounds.

SUMMARY

Endogenous and added nitrate is rapidly reduced by incubating saliva. The progressive decrease in salivary and added nitrate is accompanied by a transient accumulation (and apparent reduction) of nitrite.

The nitrate-nitrite reductase systems in saliva are present in the sediment, with components of the supernatant serving as hydrogen donors. Yeast extract, pyruvate, or lactate will replace the necessary elements of the electron donor system normally occurring in the supernatant.

The reduction of nitrate through nitrite is markedly affected by pH, with maximum activity demonstrated between 6.0 and 6.4.

Although nitrate reduction is little affected by the presence of oxygen, nitrite reduction is completely inhibited in aerated, incubating saliva.

In a limited survey of 40 young adults, no relationship between caries experience (DMF) and the capacity of the individual's saliva to reduce nitrate through nitrite was found.

The authors gratefully acknowledge the assistance of Dr. H. Roy Gravelle, director, Dental Health Section, Oklahoma State Department of Health, who clinically examined the patients studied.

After the preparation of this manuscript, an abstract of a paper (G. M. SAVOSTI'IANOV, K. Voprosu o nitratakh sliuny ["On the Question of Nitrites in Saliva"], *Fiziol. Zhur.* SSSR, **23**:159–64, 1937, in *Bibliography on Saliva* [Washington, D.C.: Office of Naval Research, Department of the Navy 1960], p. 328), describing the reduction of nitrate by saliva, came to the attention of the authors.

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Postnatal Dentofacial Changes Induced in Rats by Prenatal Riboflavin Deficiency

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In previous communications we have described dentofacial changes in the young of rats whose mothers had been fed riboflavin-deficient diets during pregnancy.[1, 2] Some of the animals which showed such congenital malformations were so severely affected that they were not viable. For instance, those with cleft palate and extreme shortening of the mandible could not suckle and died soon after birth. Occasionally in such experiments an animal that appeared almost normal at birth and proved viable grew up to show at weaning a slight retrognathia. It seemed of interest to observe the development of the dentofacial complex in such mildly affected animals. In the following pages the development of the facial bones and of the teeth of some of these animals will be described and illustrated.

EXPERIMENTAL METHODS

The maternal diet, the technique, and results of breeding animals fed riboflavin-deficient diets have been described.[3] The original method was simplified by Nelson, Baird, Wright, and Evans[4] by adding the antagonist galactoflavin to the maternal diet. This method was further modified by us by replacing the oral administration of galactoflavin with intraperitoneal injections.

The young used in this investigation were obtained from 46 pregnant rats of a commercial Wistar strain. The mothers were fed a riboflavin-deficient diet from the first to the sixteenth day of pregnancy. On either the twelfth, thirteenth, or fourteenth day they were given intraperitoneally, in 1 cc. of saline, 2 mg. of the riboflavin antagonist galactoflavin.* By this procedure a total of 219 young were obtained, of which 139 were normal and 80 abnormal. The abnormal litters were sometimes heterogeneous and contained severely affected young, as well as young that were normal on inspection except for a slightly bent tail. Six animals of the latter type were made the object of this study. Malocclusion of the incisor teeth was recognized in them after weaning at 3 weeks. These animals were placed on powdered Purina Chow, an adequate diet for growing rats. Approximately 6 weeks after birth the incisor teeth were cut about 2 mm. from the gum line. This was necessary to prevent the teeth from growing into the soft tissues of the roof of the mouth. It was noticed that in animals whose teeth were not

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cut regularly the uncut incisors were impinging upon the soft tissues of the oral cavity. Such animals showed a slight weight loss. However, when the teeth were regularly cut, the animals could ingest food, gain weight, and grow normally. They required little special care, but they had to be placed in cages with a few normal rats, since it was observed that when kept by themselves they could not clean their skin properly and became infected with lice. Their normal cagemates supported their skin hygiene.

The experimental animals were observed, photographed, and radiographed at the age of 2, 4, and 6 months and also at 18 months just prior to sacrifice. In some animals the growth rate of the incisor teeth was measured by initial marking of the tooth at the gum line and subsequent observation of the migration of the dental mark.

FIG. 1.—Mouths of two abnormal rats approximately 6 weeks of age, showing overgrowth of incisor teeth and malocclusion.

RESULTS

In previous papers[1, 2] it was pointed out that the pattern of the rat skull was modified *in utero* by prenatal riboflavin deficiency. The most striking changes occurred in the lower jaw and in the palate. Milder anomalies were observed in the nasal and premaxillary regions. The dental anomalies associated with the skeletal malformations indicated that the development of the teeth and that of the supporting skeleton are closely related. Three degrees of severity were recognized, ranging from moderate through intermediate to severe. In general, only animals classified as moderately affected survived. All the survivors had incisor teeth, although some of them were of small size, distorted in form, and confined to the premolar corpus. None of the survivors had cleft palates.

The external appearance of a severely affected newborn rat, offspring of a riboflavin-deficient mother, was illustrated and compared with a control newborn in one of our previous communications.[1] In contrast to such extreme forms of retrognathia and cleft palate, viable young could not be recognized as abnormal at birth. However, when they reached weaning age, a very slight retrognathia was noticed, and the incisor teeth appeared abnormal. At the same time it was observed that these animals could not incise and ingest the hard pellets of Purina Chow, the food given to our control rats. During the next few weeks malocclusion of the incisor teeth became manifest (Fig. 1).

In all six animals the lower incisors grew toward the left, the upper incisors toward the right of the mid-line. This abnormal course of the incisors resulted in a cross-bite. The shapes of the individual teeth were sometimes unequal, so that two incisors in the same jaw were of different diameters and different curvatures. As a result, abnormal interproximal relationships were established. Most important, however, was the fact that the upper and lower incisors did not oppose each other and did not develop a normal occlusal pattern. Owing to lack of attrition, the upper as well as the lower incisors grew backward into the oral cavity. In most of these animals the unoccluded teeth grew toward the roof of the mouth, ultimately impinging upon the palatal mucosa. As stated before, these incisors had to be cut to keep the animals alive. The growth rate of the incisors, measured by the movement of file marks from the gum line, was normal.

These external observations were supplemented by roentgenograms, cephalometric analysis, and ultimately by clearing of the skeleton. The degree of anomaly of the type of newborn young capable of survival is illustrated in Figure 2, *B*, which shows in a

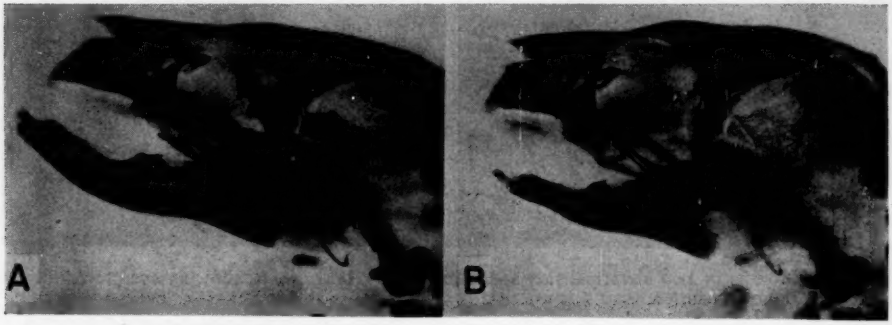

FIG. 2.—Heads of cleared newborn rats. *A*, normal; *B*, mildly affected young of riboflavin-deficient mother.

cleared specimen that the lower incisor as well as the premolar corpus of the mandible are reduced if compared with a normal newborn rat illustrated in Figure 2, *A*.

Figure 3 shows profile roentgenograms of a control animal, and Figures 4, 5, and 6 profile roentgenograms of three abnormal animals of the type that could be raised. These roentgenograms taken at ages 2, 4, 6, and 18 months compare the development of the normal and abnormal animals. The abnormal pattern present at birth (as seen in Fig. 2, *B*) was essentially maintained during postnatal growth of these rats (Figs. 4, 5, and 6), although they were reared on an adequate diet.

The facial development is illustrated by roentgenograms in Figures 3–6. Compared with Figure 3, which shows the dentofacial pictures of a control animal at 2, 4, 6, and 18 months of age, all the abnormal animals illustrated in Figures 4–6 have, in the presence of a rather normal mandibular ramus and molar corpus, a slight reduction of the premolar corpus. The reduction of the premolar corpus is possibly less marked in postnatal life than at birth (Fig. 2, *B*). In the control (Fig. 3) the premolar and molar corpora are continuous, forming a smooth curve. In all abnormal animals, the premolar and molar corpora form an obtuse angle. Generally, the osseous pattern of the abnormal animal's mandible was not significantly altered during the period of

16 months represented in Figures 4–6. The illustrations also show that the maxilla is little affected in these mildly abnormal animals.

The dental pattern illustrated in Figures 4–6 does not give a natural picture of the incisors, since they had been cut shortly before the roentgenograms were taken. Figures 4–6, *D*, are exceptional, since the incisors had been allowed to grow without interference in this final stage.

It can be seen in Figures 4–6 that throughout life the lower incisor teeth, whose intra-alveolar portions always remained small, never established normal relations to the mandible. They remained confined essentially to the premolar corpus, never reaching the region of the mandibular notch, a developmental retardation equivalent to a

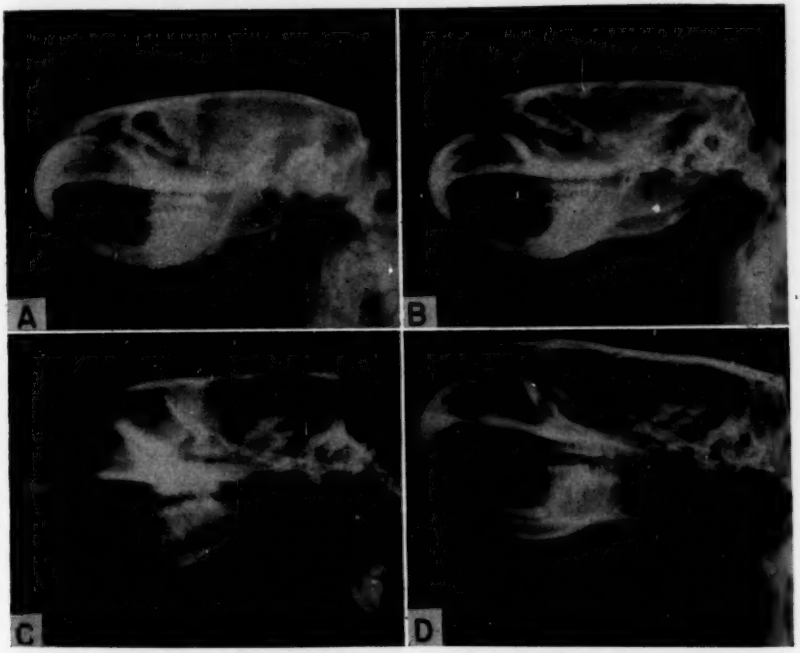

FIG. 3.—Roentgenograms of head of control animal. *A*, age 2 months; *B*, 4 months; *C*, 6 months; *D*, 18 months.

physiologic age of 5 days. The alveolar processes of the lower incisor teeth paralleled in form and mass the size and curvature of the tooth. They were always hypoplastic in the presence of rudimentary teeth. Owing to the maldevelopment of the lower incisor teeth, the upper incisors were also abnormal in growth and development. This is clearly seen in Figures 4, 5, and 6, *D*, where the uncut upper incisors curve backward into the oral cavity. While in the control (Fig. 3, *D*) the upper incisor is directed downward in an almost vertical direction, in the abnormals the teeth, being unopposed by lower incisors, take a backward direction, assuming a semicircular or almost circular shape. The lower incisors, when they did grow proximally into the molar corpus, showed irregularities of form. This is illustrated in Figure 4, *D*, and in Figure 7, where the curvature of the lower incisor is of a less than normal diameter. In Figure 7, roentgenograms of the least abnormal animals are reproduced. The lower incisor of this

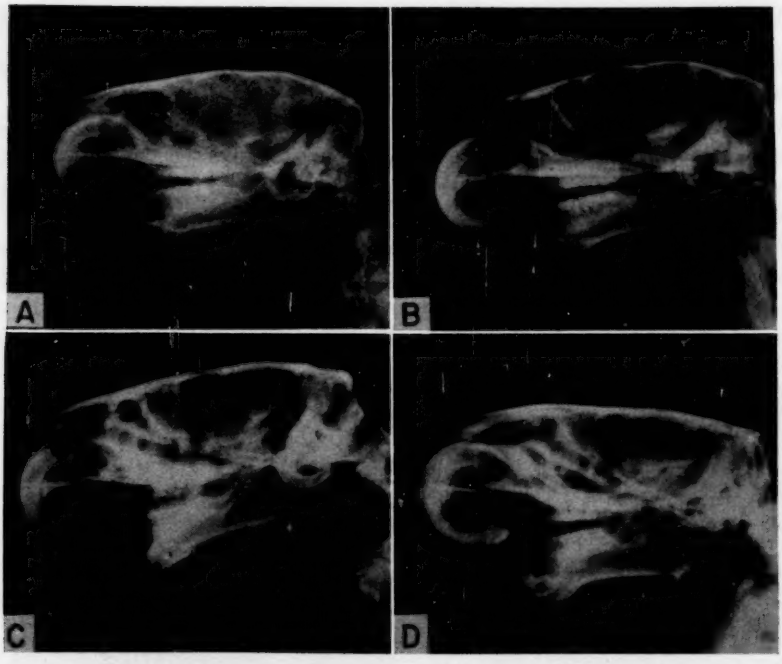

FIG. 4.—Roentgenograms of head of an abnormal animal. *A*, age 2 months; *B*, 4 months; *C*, 6 months; *D*, 18 months.

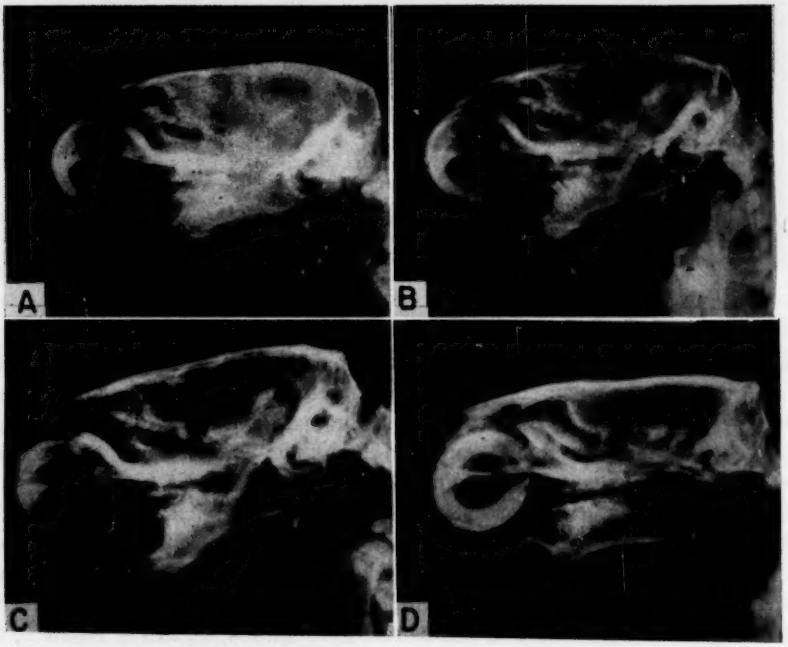

FIG. 5.—Abnormal animal. *A*, age 2 months; *B*, 4 months; *C*, 6 months; *D*, 18 months

animal appears less hypoplastic than those illustrated in Figures 4–6. The incisor reaches proximally into the molar corpus and the anterior part of the ramus. Since the curvature of this tooth has a smaller diameter than that of the control, the tooth curves abruptly forward and upward, bypassing the upper incisors like the corresponding teeth of more severely affected animals.

A different deviation from the abnormal pattern is seen in Figures 5, *D*, and 9, *B*, which were obtained from the same animal. There the lower incisor is straight and directed forward without any curvature. Irrespective of the degree of anomaly, all six animals developed a cross-bite of the incisor teeth.

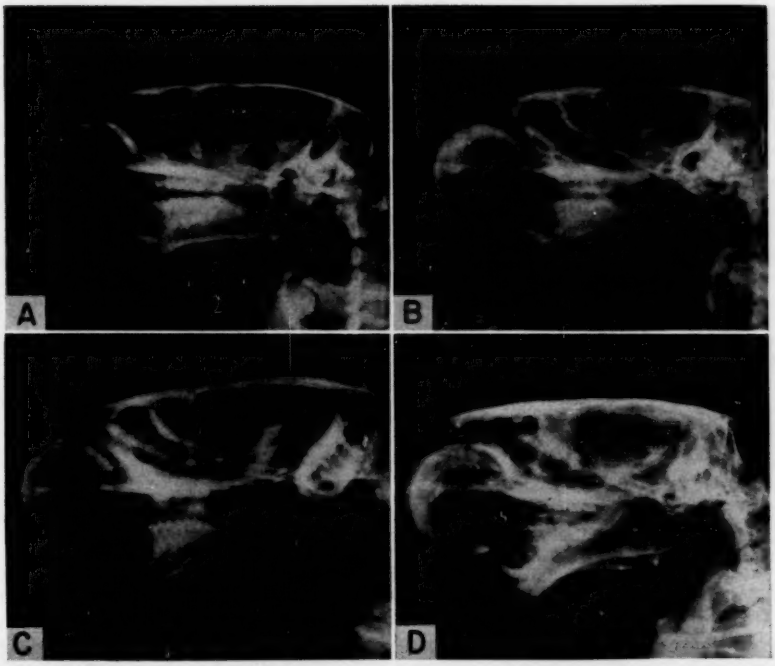

FIG. 6.—Abnormal animal. *A*, age 2 months; *B*, 4 months; *C*, 6 months; *D*, 18 months

An interesting observation was made in a seventh animal illustrated in Figure 8. In Figure 8, *A*, which was obtained at 2 months of age, the mass of the pre-molar corpus appears increased and in the roentgenogram shows a suggestion of cyst formation. In the anterior portion, the region normally occupied by the lower incisor tooth is represented by an area of reduced density. At 4 months of age (Fig. 8, *B*) the mass of the premolar corpus appears reduced, and the area of translucency has disappeared. Because of the resemblance of these pictures to certain human pathologic roentgenograms, one can probably assume that there existed in this animal cystic formations which were later replaced by osseous tissue.

The molar teeth as well as the molar corpus appear essentially normal in the roentgenograms (Figs. 4–6).

In a previous communication[2] we analyzed the facial angles of three abnormal ribo-

flavin-deficient neonates and compared them with a control animal. Maxillofacial triangles[5] were traced upon photographs, using the following three sides: (*a*) Bolton plane[6] as the cranial base line (*BN*); (*b*) facial line (*NM*) from nasion to the inferior alveolar point (*IA*); and (*c*) the mandibular line (*MB*), a tangent to the inferior border of the mandible continued anteriorly to meet the facial line at *M* and posteriorly to the cranial base line at *B*. Three additional lines were drawn from the nasion: one to the superior alveolar point or prothion (*P*), another to the anterior inferior molar point (*A*), and a third to the internal angle point of the mandible (*I*). All the angles at *N* and the angle at *B* were measured in the control and abnormal animals.

Figure 10 *A*, shows the facial angles of a control newborn, and Figure 10, *B*, shows those of a mildly affected abnormal riboflavin-deficient newborn young. It can be seen that the angular relationships are partly changed and partly unaffected by the experimental conditions. The facial angle, *PNB*, which characterizes the position of the prothion, was only slightly reduced by the retarding process. Maximal reduction was found in angle *IA–N–A*, which is a measure of the length of the premolar mandible.

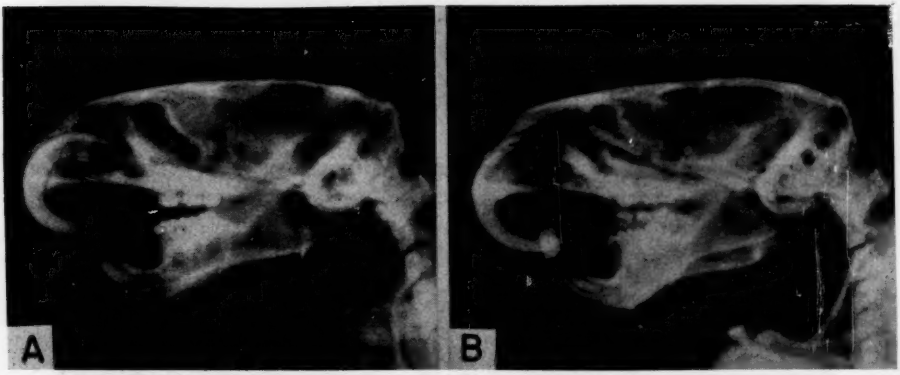

FIG. 7.—Roentgenograms of head of very mildly affected abnormal animal. *A*, age 2 months; *B*, 4 months. Note the marked curvature of the lower incisor tooth and the proximal end of this tooth.

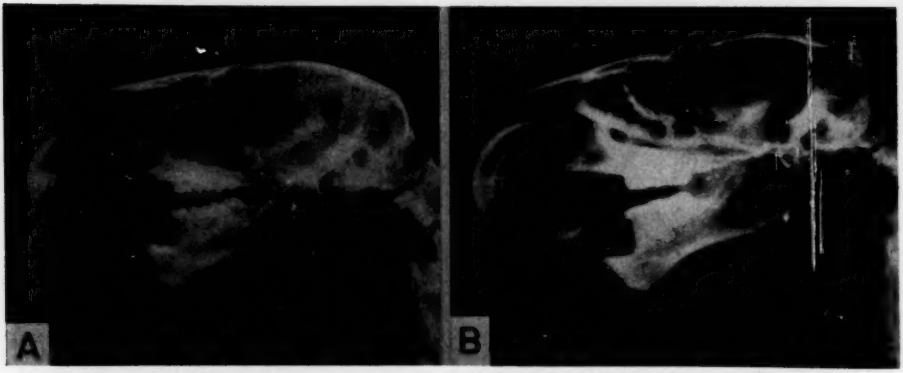

FIG. 8.—Roentgenograms of an abnormal rat. *A*, age 2 months. Observe the radiolucent areas in the premolar corpus mandibulae. *B*, 4 months. Notice the absence of radiolucent areas and change in form of premolar region.

As indicated by angle *A–N–I*, the molar corpus is markedly reduced though less so than the permolar corpus. In contrast, angle *I–N–B* is not significantly affected by the deficiency, and angle *N–B–M*, which represents the relationships of the cranial base to the inferior border of the mandible, also remains unchanged in the abnormal young.

Figures 11 and 12 illustrate continuation of the cephalometric analysis of a control and a mildly abnormal rat through months 2–6.

Figure 11, *A, B, C*, shows the facial angles of a control animal at 2, 4, and 6 months, respectively. It can be seen that in the controls the pattern obtained at 2 months is almost maintained at 4 months and 6 months, except for a very slight reduction of angle

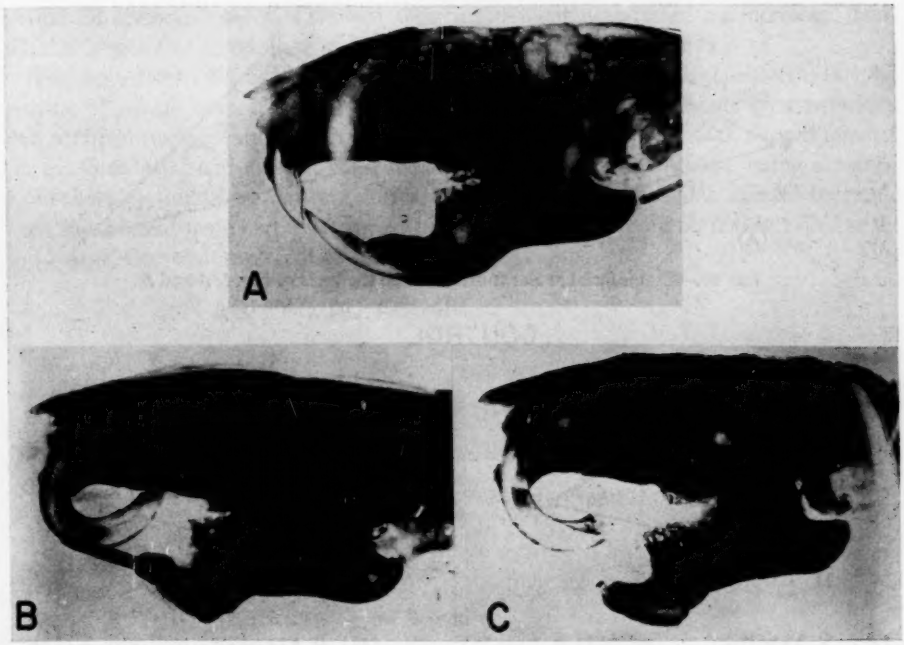

FIG. 9.—Cleared heads of rats 18 months of age. *A*, control; *B*, abnormal animal. Note that the lower incisor extends straight forward and that the upper incisor is distorted in form, overgrown, and curving toward the palate. *C*, another abnormal animal. Observe the reduced diameter of the lower incisor tooth and its confinement to the premolar corpus.

I–N–B. Figure 12, *A, B, C*, represents the facial angles of an abnormal animal at 2, 4, and 6 months. If compared with control, the abnormal animal at 2 months shows maxillary and mandibular retrognathia, as evidenced by the reduced angles *P–N–B* and *IA–N–B*. By 4 months, angle *P–N–B* has increased in the abnormal animal, and by 6 months it has enlarged to the size of that of the control of 6 months. This change, which indicates growth recovery of the maxilla, was observed in five of six abnormal animals. Angle *IA–N–B*, which represents total mandibular length, shows an interesting adjustment in the abnormal animal. At 2 months of age this angle is 71°, which is 10° smaller than that of the control of the same age. The angle then increases to 78° by 4 months, which makes it identical with that of the control of 4 months. Between

4 and 6 months this angle is reduced slightly—to 75°—which is slightly smaller than the angle of 78° characteristic of the 6-month control. All abnormal animals showed some increase of angle *IA–N–B* from 2 to 6 months. Angle *IA–N–A*, which represents a measure of the length of the premolar corpus, is reduced from a size slightly larger than that of the control at 2 months and 4 months to a size 5° less than the control 6 months. However, the reduction of this angle from 2 to 6 months was less marked in two other abnormal animals and was absent in three. Between 2 and 4 months, slight enlargement of the molar corpus represented by angle *A–N–I* occurred in the abnormal animal, and a normal size was obtained by 6 months. However, this behavior was not observed in four other abnormal animals, which showed either no change or a reduc-

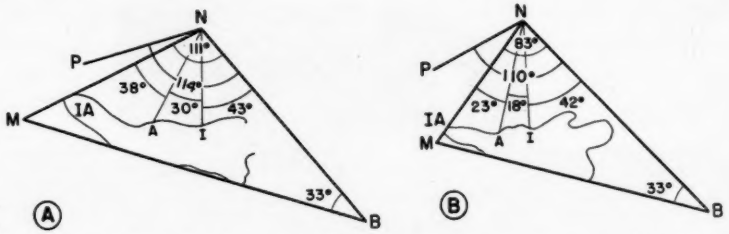

FIG. 10.—Craniofacial angles constructed on the basis of Fig. 2, *A* and *B*

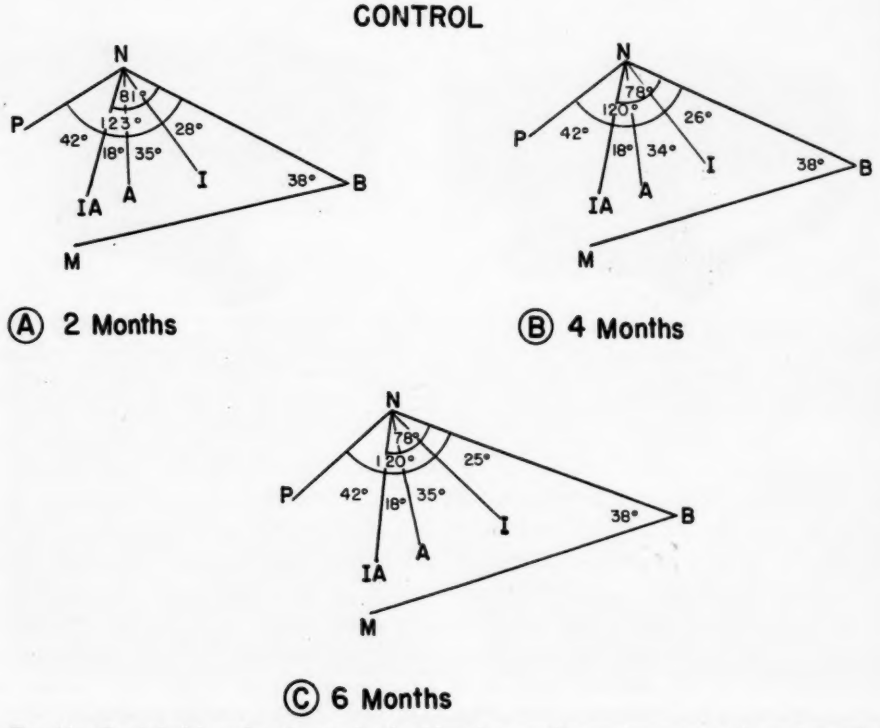

FIG. 11.—Craniofacial angles of control animal based on profile roentgenograms. *A*, age 2 months; *B*, 4 months; *C*, 6 months.

tion of angle *A–N–I* between 2 and 6 months. Angle *I–N–B*, which represents the anteroposterior dimension of the ramus, decreases in the control slightly from 2 to 6 months and increases during the same time in the abnormal animal from 21° to 28°. This increase was uniform in all abnormal animals. From 2 to 6 months there is a very slight enlargement of the cranial base–mandibular angle *N–B–M* in the abnormal animal. This angle, which records the vertical development of the ramus mandibulae, showed no regular behavior in the abnormal young between 2 and 6 months. It increased considerably in two animals, decreased in two, and remained unchanged in one. Thus angles *IA–N–B* and one of its component angles, *I–N–B*, increased with regularity in all abnormal animals between 2 and 6 months. This signifies that during this period all abnormal animals showed some adjustment manifested by increased mandibular length due to increase in anteroposterior length of the ramus.

This adjustment did not correct the severe malocclusion of the incisor teeth, but, by cutting of the incisors, ingestion of food became possible, the animals grew normally and attained sexual maturity. The four abnormal males were kept with normal females for many months, and most of these females became pregnant. All the young obtained from these matings were entirely normal and grew to maturity. One abnormal female bred to a normal male had five young which appeared externally normal but died within 17 days. One abnormal female had no offspring.

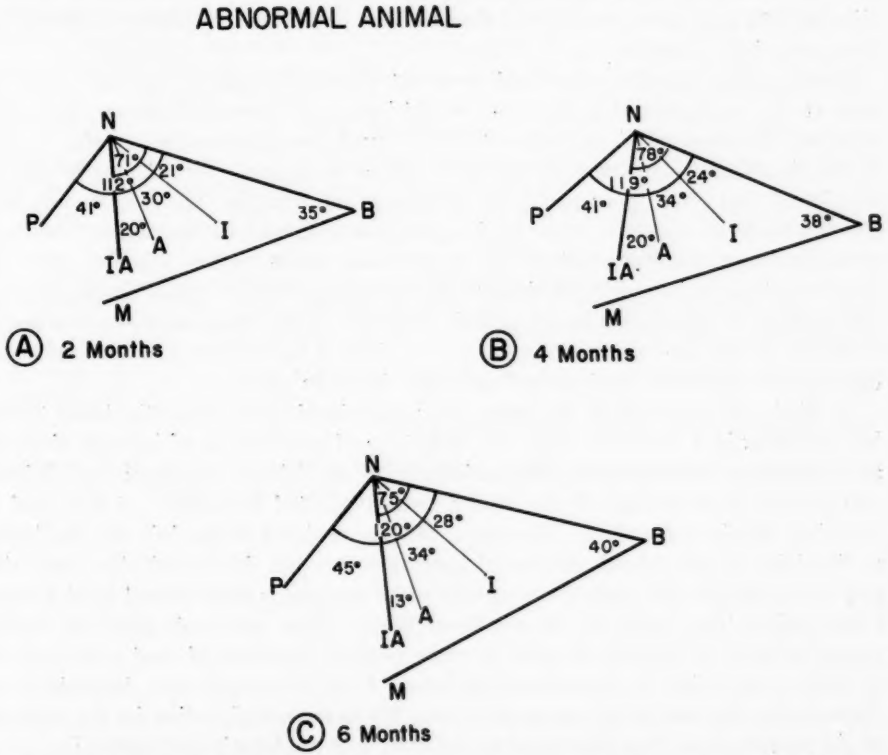

FIG. 12.—Craniofacial angles of an abnormal animal based on profile roentgenograms. *A*, age 2 months; *B*, 4 months; *C*, 6 months.

DISCUSSION

Most experiments concerned with the production of congenital malformations by prenatal environmental factors were terminated at birth of the offspring. Usually, attention was paid to the most severe deviations from the normal, which were recognizable in the newborn by inspection, clearing, or microscopic sectioning. Since most of the severely affected offspring were not viable after birth, only few investigations along these lines were extended beyond the neonatal period. Yet the fate of offspring modified by environmental adversities *in utero* is of definite interest because such animals can serve as models in investigations concerned with postnatal patterns of growth and development which have their root in prenatal life.

The results reported above were derived from experiments with rats fed a riboflavin-deficient diet and injected with the riboflavin antagonist galactoflavin. It was observed that some young which appeared normal at birth were subclinically abnormal, as evidenced by irregular development of the jaws and teeth after the third week of postnatal life. As described before, the incisor teeth had an atypical occlusal form due to malposition and lack of attrition. In these rats, which had only mildly abnormal jaws—compared with other specimens obtained by this method—the malocclusion of the incisor teeth was so severe (Fig. 1) that the animals would not have survived, had these teeth not been cut periodically. Thus even animals without manifest anomalies at birth may have a subclinical defect of the jaws which, by abnormal dentition, becomes a lethal trait.

Unfortunately, the rats described were not examined cephalometrically at birth, since it was not known then that they would become of interest. However, when the abnormal development of the incisor teeth betrayed the malformation, careful studies of the dentofacial complex were initiated. When the animals were 2 months old, it was found that both upper and lower jaws were retrognathic. The maxilla was less affected than the mandible, resulting in a maxillomandibular disproportion. This was probably the morphologic basis of the malocclusion which became manifest with the eruption of the incisor teeth. In these mildly abnormal young, eruption of the incisors was normal in time and rate of growth. However, from the time when functional occlusion should have occurred at the age of about 3 weeks,[7] the maxillomandibular disproportion persisted, and normal occlusion failed to occur.

In all six animals studied, the upper incisors deviated to the right, the lower to the left, resulting in a cross-bite (Fig. 1). This type of cross-bite is of interest, since in an observation of Stotsenburg, cited and illustrated by Addison and Appleton,[7] Wistar rats showing malocclusion of the incisor teeth attributed to softness of food had a cross-bite which was reversed. The upper incisors deviated to the left and the lower to the right. In our animals the dental anomalies were not due to soft diet, since the incisors erupted in the anomalous pattern while the young were offered hard Purina Chow pellets. The change to the powdered Purina Chow was made after the dental anomalies were recognized, in order to make possible ingestion of food and survival. It seems certain that the dentofacial deviations from the normal were determined by the maternal diet before the young were born. We have no explanation for the reversal of the pattern from that illustrated in Addison and Appleton's publication.[7]

Analysis of the incisors of these animals showed that they were not only abnormal

in position but also smaller in diameter than normal incisors. They were distorted in form and abnormally curved. These alterations were more pronounced in the mandibular than in the maxillary incisors. The lower incisors were confined in some of the animals to the premolar corpus of the mandible, a stage of development equivalent to a physiologic age of 5 days. While the incisors were functionally useless and handicapping to the animal, the molars were physiologically adequate.

In spite of limited correction of the facial anomalies by growth of the mandibular ramus from 2 to 6 months, as ascertained by a cephalometric method, the malocclusion was not corrected. Thus a mild prenatal damage of the primordia of the jaws, induced by an environmental teratogenic method, manifested itself in dentofacial anomalies from the time of eruption of the incisors to the age of 18 months, when the animals were sacrificed.

SUMMARY

Rats with mild facial anomalies produced by maternal riboflavin deficiency could be raised to maturity. Although the anomalies of the jaws were only subclinical at birth, the dentofacial deformities became manifest at the time of eruption of the incisor teeth. The anomalies of the incisors were so severe that survival was made possible only by periodical cutting of the teeth. There was a constant pattern of malocclusion. The dentofacial anomalies induced by prenatal riboflavin deficiency did not correct themselves to normal during 18 months of postnatal life, although the animals were fed an adequate diet. Young obtained from matings of these abnormal animals with normal rats of the same stock resulted in anatomically normal young.

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The Pathogenic Role of a Rat Oral Streptococcus in Incipient Dental Caries

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Recent studies on gnotobiotic rats have shown a causal relationship between an oral streptococcus and the pathogenesis of dental caries (R. J. Fitzgerald, personal communication). The streptococcus is a versatile micro-organism apparently capable of invading tissue, of elaborating biologically active cells, acids, enzymes, and toxins, and, like other organisms, of eliciting a delayed hypersensitive response. Considerable attention has been given to the significance of acidogenesis, with less consideration to the other microbial properties. It seemed desirable, therefore, to extend our definition of the relationship between this organism and the incipient carious lesion.

Rat oral streptococcus strain FA-1 secured from Dr. Robert J. Fitzgerald, of the National Institute of Dental Research, was routinely cultivated on a yeast extract–peptone-dextrose broth for 24 hours at 37° C.

Osborne-Mendel rats between 20 and 50 days of age and maintained on either Purina laboratory chow or the cariogenic diet previously reported (R. R. Steinman, C. G. Hewes, and R. W. Woods, *J. D. Res.*, **38**:592, 1959) were employed in this study. The animals were sacrificed and the jaws prepared by the method of Steinman *et al.* (*loc. cit.*) and fixed by the method of Vazquez and Dixon (*J. Exptl. Med.*, **104**:727, 1956). Incipient lesions were recognized by localized changes in ninhydrin-reactive protein, in dehydrogenase activity, and in availability of sulfhydryl radicals (Steinman *et al.*, *loc. cit.*; R. W. Woods, *J. D. Res.*, **38**:592, 1959; Steinman, R. R., *J. D. Res.*, in press).

Systemic inoculation of organisms.—Intracutaneous and intraperitoneal inoculation of forty-seven rats, 20–30 days of age, with 0.25 ml. of a washed-cell suspension had no observable systemic effect on the rats over a period of 20 days and did not alter the incidence of incipient carious lesions. Neither a systemic toxicity of the organisms nor their allergenicity appeared to influence the pathogenesis of the lesions.

Fluorescence localization of organisms.—Sections from thirty-two rats, 20–50 days of age, were stained with rabbit anti-streptococcal globulin conjugated with fluorescein isothiocyanate by the method of Marshall, Eveland, and Smith (*Proc. Soc. Exptl. Biol. Med.*, **98**:898, 1958). In addition to routine controls, specificity of staining was established on suspensions of the homologous organisms, on fresh isolates from rat oral washings, and by the failure of staining of heterologous bacteria. The sections frequently exhibited fluorescence referable to the organisms in the debris of the grooves, rarely showed fluorescence in the superficial layers of the enamel at the bases of the grooves, and never in the underlying dentin. Penetration of these organisms did not discernibly occur in these early lesions.

In vitro application of lysate.—In view of the fact that a lysate of this streptococcus has been shown to possess pronounced biological activity (J. D. Zwemer and R. R. Steinman, *J. D. Res.*, in press), it seemed appropriate to test such a preparation on intact murine dentinal tissue. Lysate prepared by the method of Stetson (*J. Exptl. Med.*, **104**:921, 1956) was incubated with the sections for 2 hours, and then the sections were examined for dehydrogenase activity and sulfhydryl availability. Control observations were made on sections exposed to buffered saline and to the alkaline saline supernatant of homogenized glass beads. Sections from more than one hundred rats consistently indicated a specific, marked diminution in dehydrogenase activity and diminished availability of sulfhydryl radicals. These generalized histochemical changes were identical with those observed locally in the natural lesion. Efforts are now being made to characterize the streptococcal component(s) involved and the exact nature of the affected dental substrate.

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Retention of Carbohydrate from Sugar Solutions by Salivary Sediment

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Several investigators have observed that momentary treatments of dental plaque or salivary sediment with sugar solutions will bring about an increase in glycolysis that persists for 2–6 hours. This observation is puzzling because it is difficult to explain how the small quantity of sugar that diffuses into sediment in 3 minutes is able to depress the pH level for 120 minutes. Sugar in the microbial film can be lost both through diffusion and through utilization by micro-organisms. Possibly the action persists because sugar is coverted to a less diffusible form, such as polysaccharide, mucoprotein, or phosphate esters. Snyder and others (*J. D. Res.*, **34**:368, 1955) have reported that sucrose can be converted to levan within 5 minutes by oral micro-organisms. This polymer is water-soluble but would be less diffusible than the parent structure, sucrose. The decrease in diffusion rate might serve to hold the substrate at a site which would make it available for glycolysis by the micro-organisms.

This question was approached by means of anthrone tests on salivary sediment before and after treatment with sucrose solutions. One-half-ml. aliquots of homogenized saliva were centrifuged at 2400 r.p.m. for 20 minutes and the supernatant decanted. One aliquot was refrigerated; another was treated with 30 microliters of M/2 sucrose solution and then refrigerated; and the third and fourth aliquots were treated with the same volume and concentration of sucrose and allowed to incubate for 20 and 45 minutes, respectively. All samples were extracted three times with stirring and centrifugation, using 5 ml. of absolute alcohol to remove sucrose. Next 2 ml. of water was added, and the carbohydrate remaining in the sediment was determined by the anthrone modification used by Donald J. Beck (Master's thesis, University of Rochester, 1959).

The investigation comprised 4 aliquots of pooled sediment with 10 or 11 replications. The mean per cent transmission of the control samples after reaction with anthrone was $23 \pm 3\%$, equivalent to 76 μg. of sucrose. The samples treated with sucrose at 4° C. gave an average transmission of $21 \pm 4\%$, equivalent to 80 μg. of sucrose. Samples incubated at 37° C. for 20 and 45 minutes were 14 ± 4 and 8 ± 2 per cent transmission, respectively, corresponding to 100 and 166 μg. In ten out of eleven comparisons the incubated samples showed a higher carbohydrate content than did the refrigerated sample. In all eleven comparisons the 45-minute sample showed more carbohydrate than did the 20-minute sample. These results indicate that salivary sediment has the capacity to convert sucrose into a substance which is alcohol-insoluble, at a rate approximating 10 per cent of the sediment weight per hour. This capacity is dependent on temperature, for it is less at room temperature (data not given) and practically lost at 4° C. Samples varied in their ability to retain carbohydrate. A single water extraction of the alcohol-extracted sediment removed much of the anthrone-positive material, indicating that it is water-soluble. We assume that micro-organisms have the capacity to store carbohydrate from sucrose solution in some non-diffusable form, such as polysaccharide, mucoprotein, or phosphate esters.

Alcohol-insoluble carbohydrate has also been found in each of 13 samples of dental plaque obtained from the teeth of subjects who refrained from brushing for 3-4 days. The amounts present were uncertain, chiefly because of difficulties in measuring the quantity of dental plaque, but 5 of the samples appeared to approach 10 per cent carbohydate.

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Effect of Diet on Alveolar Bone Resorption

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As a result of recent studies on the cariogenic potential of corn, wheat, oats, and rice, Dodds has called attention to the fact that a diet containing ground whole oats was responsible for "abnormally developed mandibular bones characterized by an extensive outward bowing of the shelf of the bone where the teeth are attached" (M. L. Dodds, *J. Nutrition,* in press). In a similar study relative to the caries potential of whole wheat, whole oats, buckwheat, and millet we have observed an unusually striking effect of the oat-flour diet on the gingivae and the alveolar bone of white rats.

This oat-flour diet, which was similar to our other cereal diets except for the kind of cereal, was as follows:

	Per Cent		Per Cent
Whole-oat flour	76.55	$CaCO_3$	1.25
Glucose	18.00	NaCl	1.00
Liver powder	2.00	Sodium phytate	1.20

A vitamin supplement was given orally so as to provide 1,500 units of A, 100 units of D, and 0.5 mg. of E, each week. Sodium phytate was added as a source of phosphorus in an organic form, to be compared with inorganic phosphate supplements as regards their cariostatic effect (F. J. McClure, *J. D. Res.*, **38**:776, 1959). The diet was fed for 90–98 days to thirty-five 21-day-old rats weighing initially 30–35 gm.

FIG. 1.—Loss of alveolar bone in rats given oat-flour diet. Normal rat mandible at far right

A gross examination of the rats, following sacrifice, revealed the presence of gingival pockets impacted with hair and food debris. Especially striking was an almost complete resorption of alveolar bone on the lingual aspect of both maxillary and mandibular molars. The bone loss in some cases extended almost to the apices of the roots, and some root resorption had occurred. There was considerable impaction of hair and food debris between the teeth. All the rats fed the oat diet were quite similar in these respects. Alveolar bone resorption is illustrated in Figure 1.

The special property of the diet causing this effect is speculative. According to our experiments and those reported by Dodds (*loc. cit.*), no such dramatic changes were present in rats fed similar diets containing whole wheat, buckwheat, corn, rice, and millet, all ground to a flour consistency. The possibility that phytate is a factor is open to some consideration, since this compound is present generally in larger amounts in oats than in most other cereals (E. Mellanby, *A Story of Nutrition Research* [Baltimore: Williams & Wilkins Co., 1950]). Although 1.41 per cent of sodium phytate was added to the whole-wheat flour and millet diets, the total phytate in these diets may not have been equal to the total phytate in the diet containing oats plus the added sodium phytate. On the other hand, it may be noted that no phytate was added to the oat-flour diet in the experiments reported by Dodds (*loc. cit.*). In addition, the phytase activity of different cereals may vary (Mellanby, *loc. cit.*), or other factors in whole-oat flour may be involved. There was some fibrous material in our oat diet due to difficulty in grinding the whole-oat grain to a flour. This condition of our diet may have contributed initially to the gingival lesions. In previous studies with both the mouse (P. N. Baer and J. Lieberman, *J. D. Res.*, **39**:215, 1960) and the rice rat (A. M. Auskaps, O. P. Gupta, and J. H. Shaw, *J. Nutrition,* **63**:325, 1957), similar lesions in the gingivae have been attributed to hair impaction.

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Use of Radioactive Enamel Solubility Studies

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A preliminary study has indicated that radioactive enamel offers a very accurate and convenient method of measuring decalcification. Powdered enamel (100–200 mesh) was exposed to a neutron flux of 1×10^{11} n/cm^2/sec for 1 week in the Oak Ridge Graphite Reactor. By an (n, γ) reaction, some P^{31} in the enamel is converted to P^{32}, which decays (half-life, 14.3 days) by emission of a 1.71-Mev. β particle to stable S^{32}. Disintegration of P^{32} provides a rapid and accurate means of following enamel dissolution.

A 2-π gas-flow Geiger counter with ultra-thin window and a decade scaler were employed to determine activities of enamel samples. Using a standard solution of radioactive enamel in HCl, a conversion factor (counts per minute to mg. of enamel) was determined, and from this the weights of

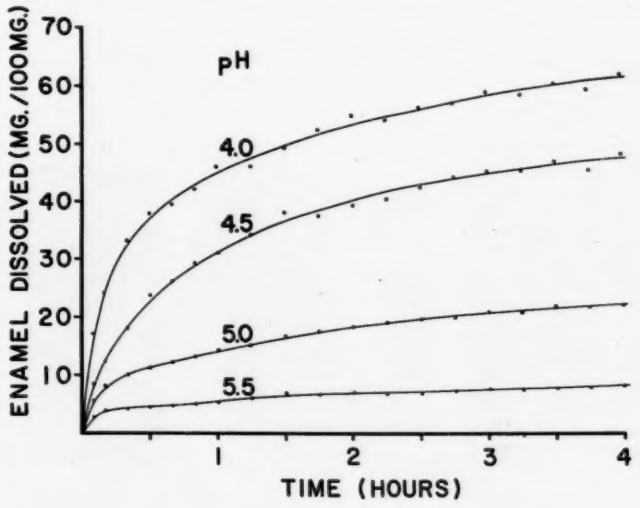

FIG. 1.—Enamel dissolved (mg. per 100 mg. total enamel) in 4 hours' agitation in 0.1 M sodium acetate buffers (50–60 mg. enamel in 25 ml. buffer) at pH 4.0, 4.5, 5.0, and 5.5 (25° C.)

enamel in the unknown samples were calculated. Enamel samples in the range of 50–60 mg. were placed in 25 ml. of 0.1 M sodium acetate buffers at pH 4.0, 4.5, 5.0, and 5.5 at 25° C. under continuous agitation. At intervals over a 4-period, 100 λ aliquots were removed and washed through a Millipore filter (120 μ pore size) using a Luer-Lok syringe with a Swinny hypodermic adapter. The filtrates were heated to dryness and counted.

Figure 1, showing the weight of enamel dissolved plotted against time, demonstrates that, although most of the enamel dissolution occurs in the first 30 minutes, the reaction is still proceeding slowly after 4 hours. The curves appear to be approximately asymptotic.

A possible limitation in the technique is currently being investigated. It is possible that during neutron bombardment a small fraction of the calcium phosphate in the enamel is changed to a different chemical species and that this new species may have different solubility properties from the normal apatite. However, as the results recorded here show solubilities of orders of magnitude similar to those obtained by others, using gravimetric techniques, this would not appear to be a serious practical problem.

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Identification of Decalcified Areas by Autoradiography of Neutron-bombarded Tooth Sections

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When teeth are exposed to a slow neutron flux, some of the stable P^{31} is converted to radioactive P^{32}, which decays by emission of a high-energy β particle. It was thought that the lowered phosphorus concentration in decalcified areas in tooth sections would be reflected quantitatively in autoradiographs of neutron-bombarded tooth sections.

Undecalcified 120-μ sections of carious teeth were exposed to a slow neutron flux of 1×10^{11} n/cm^2/sec for 1 week in the Oak Ridge Graphite Reactor, and then placed on nuclear track plates (Kodak NTB-2) under pressure, exposed for 15 hours, and the plates developed in Kodak D-19 for 4 minutes. Soft X-rays of the same sections were made with a Picker unit (copper target Machlett tube, 18 kv., 15 Ma.) using Kodak 649-0 spectroscopic plates, a target-film distance of 20 cm., and a 9-minute exposure. The autoradiographs and the soft X-radiographs were compared on the basis of delineation of decalcified areas and sharpness of image.

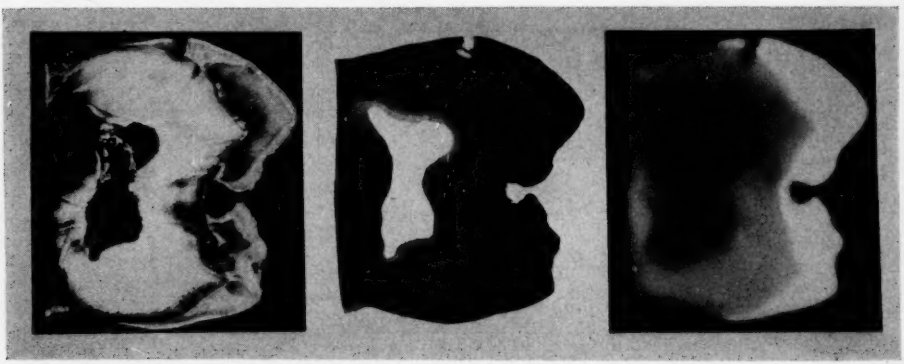

FIG. 1.—Photograph, soft X-radiograph, and autoradiograph of a neutron-bombarded tooth section. Degree of mineralization of different areas of the section is demonstrated in both the soft X-radiograph and the autoradiograph.

A reflected light photograph and prints of the soft X-radiograph and the autoradiograph of a typical section are shown in Figure 1. The areas of decalcification are as readily recognizable on the autoradiograph as on the soft X-radiograph. However, because of the high energy of the P^{32} β particle (1.71 Mev.), the definition of the autoradiograph is not comparable to that of the soft X-radiograph and, because of the nature of the isotope produced, is unfortunately optimum for neutron-bombarded sections.

However, the definition of autoradiographs is greatly improved when lower-energy β-particle emitters are employed. It should be possible to exchange stable atoms in a tooth section with radioisotopes of low β-particle energy, such as Ca^{45} or H^3, and obtain autoradiographs of good definition which would demonstrate decalcification on a quantitative basis. Such a technique would be simple and would not require access to either a soft X-ray machine or a neutron source.

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Investigation of the Usefulness of Neutron Activation Analysis for Studying Trace Elements in Saliva

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Neutron activation analysis provides a rapid and accurate method of detecting and quantitating trace elements in biological and other materials. It was applied in a preliminary, qualitative manner to human saliva to determine the suitability of the technique for this and similar investigations. Ashed human saliva was exposed to a slow neutron flux (4×10^{11} n/cm^2/sec for 1 hour), which, by an (n, γ) reaction (G. S. Nixon and H. Smith, *J. D. Res.*, **39**:514, 1960), rendered some atoms in the ash radioactive. The gamma-ray emission spectrum of the activated sample was recorded as characteristic peaks at various gamma-ray energies (Fig. 1, total sample), using a single-channel scintillation spectrometer (employing a 2-inch NaI crystal) and chart recorder. The pronounced sodium-24 gamma emission prevents the identification of many of the other elements present, as was the experience of investigators using neutron activation analysis techniques on blood (R. P. Spencer, T. G. Mitchell, and E. R. King, *Am. J. Roentgeriol., Radium Therapy and Nuclear Med.*, **79**:1053, 1958). An attempt

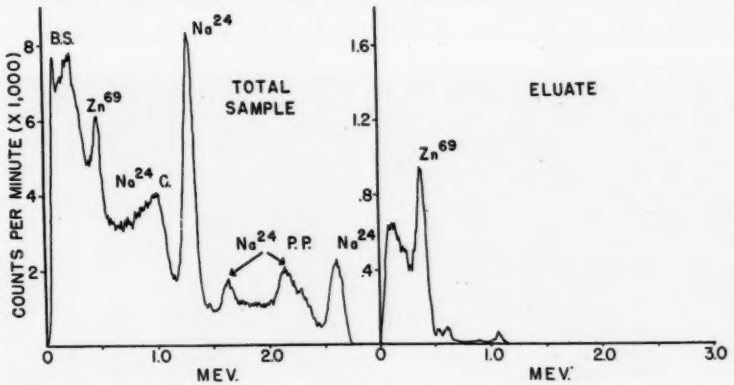

FIG. 1.—Gamma-ray spectra of neutron-bombarded saliva (counts per minute plotted against energy in million electron volts). Total sample on the left, showing masking by Na^{24}. Aqueous eluate of Dowex-1 column on the right, showing typical Zn^{69} peak. (*B.S.* = backscattering; Na^{24} *C.* = sodium-24 Compton scattering; Na^{24} *P.P.* = sodium-24 pair peaks.)

was therefore made to examine one of the more prominent trace-element peaks—that of zinc—by removal of the sodium, using a Dowex-1 ion-exchange column. Following elution with suitable normalities of HCl, an aqueous eluate was collected, and this showed a gamma-ray peak of 0.4 Mev. (Fig. 1, eluate), a half-life of 15 hours, and, by feather analysis, a maximum β energy of 0.8 Mev., confirming the presence of Zn^{69}.

If, in future work of this type separatory techniques such as ion exchange or chemical separation using carrier ions are used, it should be possible to detect submicrogram quantities of many of the elements previously reported in saliva (e.g., Na, K, Mg, Cu, Co, S, Mo, Ni, Br). Accurate quantitation of these elements is possible by means of comparative standards irradiated with the unknown. Because of its sensitivity, speed, and convenience, the method deserves wider application in dental research involving trace elements in saliva, enamel, foodstuffs, and soils.

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Glycolysis Inhibition with Arylalkanols

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Numerous compounds have been examined by the writer for ability to inhibit glycolysis that occurs in thin films of salivary sediment that have been treated with glucose solutions. Most of the papers on this topic have appeared in this *Journal* (**33**:561; **35**:591; **36**:75; **37**:798; **38**:356). The subjects covered included description of the method and its precision and studies on compounds that might contain halogen and/or ether, ester, phenol, or alcohol groups. The purpose of these investigations was to select compounds which might have potentialities as cariostatic agents, a hope which has recently been confirmed to some extent by the promise shown by three out of four compounds, tested as cariostatic agents by feeding studies with rats (Hein and Manly, presentation before International Association for Dental Research, March, 1960).

The arylalkanols are an example of the specificity occasionally found among certain types of compounds.* Inhibitory action occurs among arylalkanols containing 8–14 carbon atoms, with substitution on the benzene ring and/or the side chain. Twelve active members of this series, all derivatives of benzyl or phenethyl alcohol, are presented in the table, along with their recovery of activity after a treatment with the test chemical. All chemicals were dissolved at 1 per cent concentration in 50 per cent propylene glycol and permitted to have a contact time of 15 minutes with salivary sediment. The extent of recovery after this treatment was calculated from two differentials in pH between the solution and the thin layer of salivary sediment, one obtained before and the other after contact. Recovery is inversely related to the degree of inhibition, in that complete inhibition would correspond to zero recovery. Details of the method and calculations are presented elsewhere.

No.	Compound	First Replicate*	Second Replicate*	Control
1.....	*m*- and *p*-Methyl-phenethyl alcohol	32	20	95
2.....	*x*,*x*-Dimethylphenethyl alcohol†	16	15	108
3.....	*x*,*x*-Diethylphenethyl alcohol†	36	31	117
4.....	*p*-Chlorophenethyl alcohol	56	58	110
5.....	β-Ethylphenethyl alcohol	46	23	95
6.....	4-Methyl-4-phenyl-2-pentanol	34	36	103
7.....	2,5-Dichlorophenethyl alcohol	4	6	...
8.....	α-Propylbenzyl alcohol	36	41	95
9.....	2,4-Dichloro-α-methylbenzyl alcohol	24	17	109
10.....	2,5-Dichloro-α-methylbenzyl alcohol	51	58	95
11.....	3,4-Dichloro-α-methylbenzyl alcohol	19	12	96
12.....	*p*-Isopropyl-α-methylbenzyl alcohol	19	19	...
13.....	Phenethyl alcohol	91	90	95
14.....	α-Methyl benzyl alcohol	100	98	108

* Values are percentage of control differential.

† The alkyl substitution is on the phenyl ring; the position of the alkyl substitutions has not been determined.

Tests were also performed in 20 per cent propylene glycol with *p*-ethylphenethyl alcohol and with compounds 1 and 5 in the table. In the presence of this lesser amount of propylene glycol, compound 5 was inhibitory, but others were also active. Some degree of dilution could be tolerated, since 0.5 per cent solutions of compounds 9, 11, and 12 were active in 50 per cent propylene glycol. Certain changes in structure caused loss of activity, since two related tertiary alcohols were not active.

It appears that the particular substitutions shown for 12 of the compounds in the table have greatly enhanced the activity of the compounds over that possessed by the parent structures, phenethyl and α-methyl benzyl alcohol.

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* These compounds are the subject of a patent, U.S. 2,850,410, September 2, 1958.

Possible Etiologic Factors in Dental Erosion

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Twenty-five years ago the complaint was received from a woman patient of one of the authors (Rost) that the edge of her vulcanite denture had become sharp at the periphery. Five years later she presented the replacement with the same complaint. Six years after this the same thing had occurred to an acrylic denture that had been substituted for the original one. At this time the buccal flanges of the denture revealed many finely etched lines running distally from the cuspid to second molar area and parallel to the occlusal plane (Fig. 1). Lines were also present in the interspaces of the teeth. These ran vertically or obliquely to the sharp margin of the plate. On the surface of the denture facing the palate, other well-defined lines followed the course of the ridge, and the outline of the air chamber was all but obliterated.

Similar observations were made on the sister of this patient several years later and have been found on a number of other patients' dentures. The lines have also been found on the lingual flanges of lower dentures near the periphery, on the labial side of the flange as well as on acrylic incisors, but none

FIG. 1 (*left*).—Denture showing lines of erosion. FIG. 2 (*right*).—Erosion on fillings

have been seen on the tongue side of the upper plate. All such acrylic dentures had become thin and, wherever they covered prominences, almost transparent.

The above observations suggested some sort of abrasive action by the tissues that lay in contact with the dentures, and the thought of erosion presented itself. More than one of these individuals had been patients of one of the authors (Rost) for a long period of years, and the records of these revealed a history of erosion of their natural teeth. Further evidence was uncovered in other cases.

In several cases where amalgam or gold fillings had been placed for the express purpose of repairing erosion lesions, the lines were found on the fillings (Fig. 2). In one case they were found only 8 months after an acrylic filling had been placed in the center of the labial surface of an upper central incisor for the repair of a small congenital defect. Most surprising of all were the relatively deep grooves found on the labial surfaces of upper incisors and canines with intact enamel.

It is suggested that the primary cause of these lines is hyperactivity of the soft tissue environment of the involved structures, whether natural or artificial, and that such environmental abrasion might be one of the causes of dental erosion.

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The Effect of Tissue Extracts on the Growth of Certain Bacteria

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During microbiological assays of aqueous extracts of human gingival tissue for specific vitamin activity, Nevin, Appleman, and Kurtz noted that some samples inhibited the growth of the assay organism (*J. D. Res.*, **37**:427, 1958). In order to learn more about the relationship between tissues and their indigenous flora, aqueous extracts of several animal tissues, human skin, and normal and diseased human gingival tissue were prepared and tested qualitatively for their effect on the growth of 5 frequently encountered human oral bacteria, under aerobic and anaerobic conditions. The auxanographic method used was as described (*J. D. Res.*, **37**:427, 527, 1958) except that the test medium was modified by substituting casamino acids for the casein hydrolyzate and 0.5 per cent yeast extract for the vitamin supplement. When the *Veillonella* strain was tested, the modified medium was supplemented with 0.1 per cent sodium lactate. The results are shown in the table:

THE EFFECT OF VARIOUS TISSUE EXTRACTS ON THE GROWTH OF CERTAIN BACTERIA*

Tissue	No. of Samples	Organism									
		Veillonella FIS		*Staphylococcus* SA33		*Streptococcus* 22–32		Diphtheroid 22–5A		*Lactobacillus* 7469	
		Aer.	Anaer.	Aer.	Anaer.	Aer.	Anaer.	Aer.	Anaer.	Aer.	Anaer.
Human gingivae	16	−	−	−	−	0	0	+	0	0	0
Human skin	1	−	−	−	−	0	0	0	0	0	0
Monkey gingivae	3	−	−	−	−	+	+	0	0	0	0
Monkey duodenum	3	−	−	−	−	+	+	0	0	0	0
Monkey skin	3	−	−	−	−	+	0	0	0	0	0
Monkey liver	3	0	0	+	0	0	0	0	0	0	0
Monkey heart	3	0	0	+	0	0	0	0	0	0	0
Monkey spleen	3	0	0	0	0	0	0	+	0	0	0
Monkey kidney	3	0	0	+	0	0	0	+	0	0	0
Rat palate	3	0	0	0	0	0	0	+	0	0	0
Rat liver	3	0	0	+	+	0	0	+	0	0	+
Rat heart	3	0	0	+	0	0	0	+	0	0	0
Rat spleen	3	0	0	+	0	0	0	+	0	0	0
Rat kidney	3	0	0	+	0	0	0	+	0	0	0

* The symbols in the columns have the following meanings: − = inhibited; + = stimulated; 0 = no evident effect.

There is an indication that the type of tissue may have some bearing on the growth of organisms, since only extracts of gingivae, skin, and duodenum were inhibitory under aerobic and anaeroboc conditions, whereas the *Veillonella* was unaffected and the *Staphylococcus* was stimulated under aerobic conditions by extracts of internal organ tissues. The stimulation was manifested as zones of dense growth, up to 2 cm. in diameter, around the disks. The growth of the diphtheroid was affected similarly. Since 16 instances of growth stimulation occurred during aerobic incubation as compared with 4 during anaerobic incubation, it is evident that the extracts contained diffused reducing substances.

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